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Infection of macaques with an R5-tropic SHIV bearing a chimeric envelope carrying subtype E V3 loop among subtype B framework

M. Kaizu[1,5,6], **H. Sato**[2,3], **Y. Ami**[4], **Y. Izumi**[1,5], **T. Nakasone**[1,5], **Y. Tomita**[2], **K. Someya**[1], **Y. Takebe**[3], **K. Kitamura**[6], **O. Tochikubo**[6], and **M. Honda**[1,5]

[1]Vaccine Research and Development Group, AIDS Research Center, NIID, Tokyo, Japan
[2]Division of Molecular Genetics, Tokyo, Japan
[3]Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, NIID, Tokyo, Japan
[4]Division of Experimental Animal Research, NIID, Tokyo, Japan
[5]Development of HIV/AIDS Vaccine for HIV-1 Subtype E Project, Japan Science and Technology Corporation (JST), Kawaguchi, Japan
[6]Department of Public Health, Yokohama City University School of Medicine, Yokohama, Japan

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Summary. To establish simian/human immunodeficiency virus (SHIV) clones bearing a chimeric envelope carrying subtype E V3 loop among subtype B envelope, four subtype E V3 sequences were substituted into $SHIV_{MD14}$, a SHIV clone bearing an envelope derived from a CXCR4 (X4)/CCR5 (R5)-dual tropic subtype B HIV-1 strain. SHIV-TH09V3, an only V3-chimera clone capable of replicating in human and macaque peripheral blood mononuclear cells (PBMCs), was propagated in pig-tailed macaque PBMCs and in cynomolgus macaque splenic mononuclear cells. The propagated virus stocks were intravenously inoculated into respective macaque species. SHIV-TH09V3 infected both macaque species as shown by plasma RNA viremia, isolated viruses from PBMCs and plasma, and antibody production against viral proteins. To assess how the substituted V3 sequence affected coreceptor usage, SHIV-TH09V3 stocks propagated *in vitro* and after isolation from macaques were verified for their corecepor usage by GHOST cells assay. SHIV-TH09V3 maintained R5-tropic phenotype both *in vitro* and after isolation from macaques, in contrast to the X4/R5-dual tropic $SHIV_{MD14}$. This indicates the substituted V3 sequence among the backbone of $SHIV_{MD14}$ governs coreceptor usage. Future study of infecting macaques with SHIV-TH09V3 and $SHIV_{MD14}$ will focus on differences of the outcome caused by the different V3 sequences in connection with coreceptor usage.

Introduction

Multiple genetic subtypes of *Human immunodeficiency virus 1* (HIV-1) strains, now classified mainly into A through K [21], have been spreading and intermingled worldwide. Despite their distribution variety, only subtype B, originally from Europe and North America, has been accepted major focus of research.

For prophylaxis/vaccine development, making of macaque AIDS model is a prerequisite [1, 18]. Though several groups have so far established simian/human immunodeficiency viruses (SHIVs) carrying a whole envelope sequence from non-subtype B HIV-1 [4, 13, 16, 17], it was initially difficult to establish a usable SHIV because candidate SHIVs often failed to infect macaques. Assuming that a non-subtype B envelope as a whole does not easily fit the construction of an infectious SHIV clone, we selected V3 loop region out of subtype E envelopes for substitution into a SHIV bearing a subtype B envelope framework.

Selecting V3 region came from a concept that V3, at least of subtype B, is a relatively independent functional region governing cell tropism [9, 10, 25, 28, 31] and coreceptor usage [3, 5, 6, 29]. One concern was that amino acid sequences of subtype E V3 differed from those of subtype B as much as 50% [11, 15, 23, 34], possibly hindering subtype E V3 from enjoying the relative independence among a subtype B envelope framework. But this concern was considerably cleared by a study showing that subtype B HIV-1 clones chimeric with subtype E V3 maintained phenotypes of the V3 in terms of cell tropism and coreceptor usage [24]. This suggested the cross-subtype independence of V3 function, encouraging us to construct subtype E V3-chimera SHIVs.

Here, we report an establishment of a SHIV clone, designated SHIV-TH09V3, bearing a chimeric envelope with subtype E V3 among subtype B framework. SHIV-TH09V3 infected both pig-tailed and cynomolgus macaques maintaining R5-tropic phenotype dictated from the substituted subtype E V3.

Materials and methods

Construction of the recombinant DNA clones of V3-chimera SHIVs

pMD14 [26], a SHIV clone bearing a subtype B HIV-1 envelope derived from an X4/R5-dual tropic strain (HIV-1_{DH12}), was used as a backbone to generate V3-chimera SHIVs. Although there were two versions of pMD14 (pMD14YE and pMD14RQ) with minor differences, only pMD14YE was used in this study. Four subtype E V3 sequences, TH09V3, NH2V3, KH005V3 and NH1V3, were used for the chimeric substitution (Fig. 1A) as described previously [24]. Briefly, *Bgl* II-to-*Bsu*36 I DNA fragment (269 bp) encoding subtype E V3 and DH12 flanking sequences was generated by the overlap extension method [14], digested by *Bgl* II and *Bsu*36 I, and cloned back into pMD14 (Fig. 1B). The structures of the reconstituted regions were confirmed by DNA sequencing.

Western blot

HeLa cells (6×10^5 cells) were grown in 10% FBS-DMEM in a T25 flask for one day, and transfected with 3 μ g of the SHIV plasmid DNA using FuGENE 6 transfection reagent (Roche Diagnostics). The cells were harvested at 48 hours after transfection, washed with

PBS, and lysed on ice for 10 min with 200 μl of RIPA buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1 mM PMSF, 1 μg/ml antipain, 1 μg/ml leupeptin and 1 mM iodoacetamide). The lysates were sonicated at 4 °C for 30 sec, followed by centrifugation at 4 °C. The supernatants were recovered and the protein concentrations were quantified by Bradford protein assay kit (BioRad). 6 μg of proteins were separated per lane by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore). The membrane was incubated with antibodies described below, followed by incubation with Protein A conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The antigen bands were visualized with ECL system (Amersham Pharmacia Biothch).

The following antibodies were used: plasma of a rhesus macaque that became seroconverted by infection with SIVmac239; plasma of a positive control patient from LAV BLOT 1 kit (SANOFI Diagnostics Pasteur); plasma of two patients, J28 and NH1, who became seroconverted by infection with HIV-1 subtype E [23]; RC25 monoclonal antibody (Chemo-Sero-Therapeutic Research Institute) which recognizes HIV-1 subtype B V3 loop; rabbit polyclonal antiserum raised against synthetic peptide corresponding to the consensus V3 sequence of subtype E non-syncytium-inducing (NSI) HIV-1 from Thailand [11, 15].

Preparation of the cell-free SHIV stocks

$SHIV_{MD14}$ and V3-chimera SHIVs were prepared as described previously [24]. Briefly, HeLa cells (5×10^5 cells) were grown in 10% FBS-DMEM in a T25 flask for one day, and transfected with 30 μg of the plasmid DNA using calcium phosphate coprecipitation methods. The culture supernatants were collected at 48 or 72 hours after transfection, filtered and kept at −152 °C until analysis of reverse transcriptase (RT) activity.

Replication of the SHIV stocks in human and macaque PBMCs

0.1 ml of the SHIV stocks (2×10^4 cpm of RT activity) were incubated for 16 hours at 37 °C either with Phytohaemagglutinin (PHA, 1 μg/ml)-stimulated human PBMCs (1×10^5 cells) or with Concanavalin A (ConA, 5 μg/ml)-stimulated PBMCs (1×10^5 cells) of pig-tailed macaque (*Macaca nemestrina*). The PBMCs were washed and cultivated in 0.2 ml of 10% FBS-RPMI 1640 medium with 20 Units/ml of recombinant human interleukin-2 (IL-2) in 96-well plates. The culture medium was replaced behalf with the fresh medium every two or three days, and the collected medium was stored at −80 °C until RT activity analysis.

Preparation of the in vivo *inoculation stocks of SHIV-TH09V3*

ConA-stimulated PBMCs of pig-tailed macaque and ConA-stimulated splenic mononuclear cells of cynomolgus macaque (*Macaca fascicularis*) were infected with the SHIV-TH09V3 stock and propagated in 10% FBS-RPMI 1640 medium containing IL-2. P27 Gag concentration of the culture supernatant was determined by sandwich ELISA (Coulter). 50% Tissue culture infectious dose ($TCID_{50}$) was determined by using M8166 cells [7].

Animal care

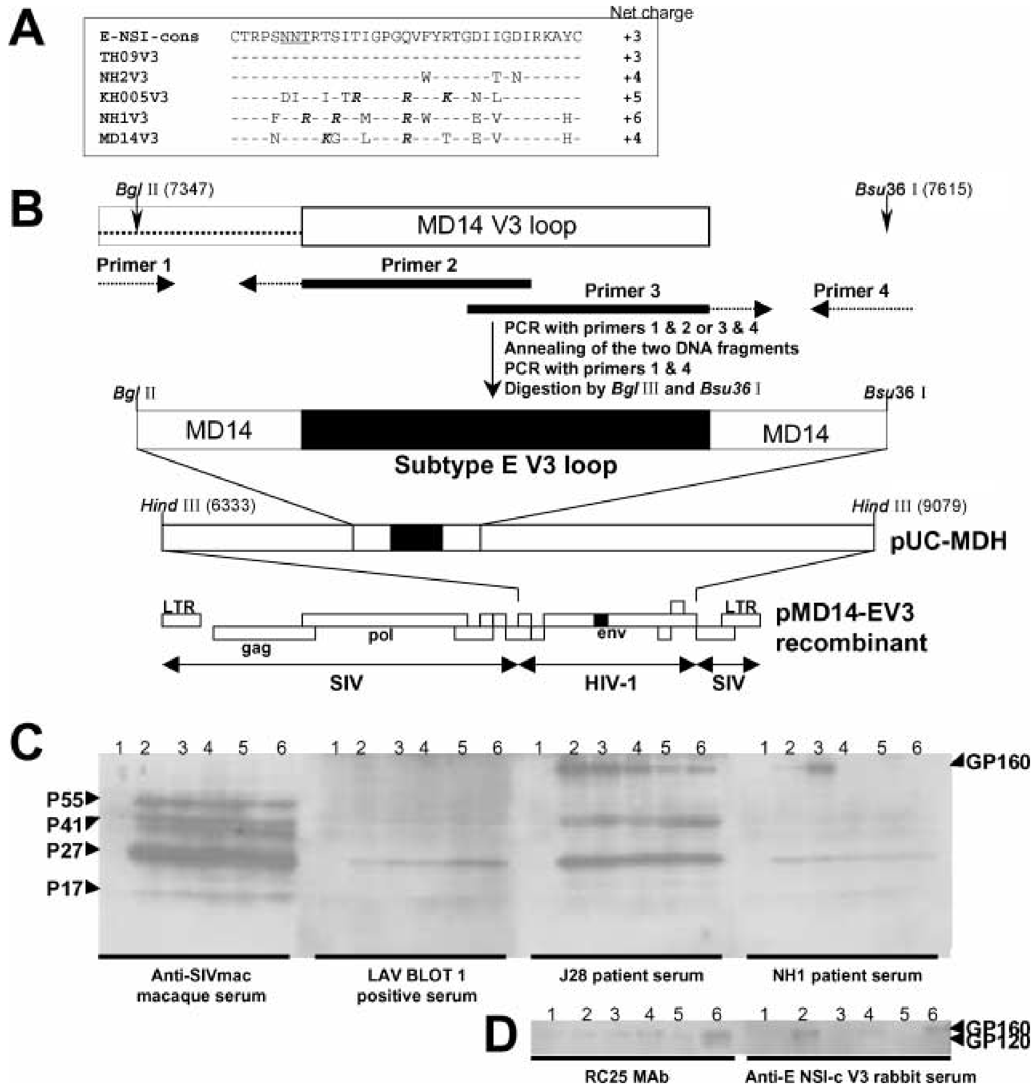
Maintenance and treatment of pig-tailed and cynomolgus macaques were strictly adhered to the guidelines of the Institutional Animal Care and Use Committee of the National institute of infectious Diseases, Japan. All the experiments were conducted in accordance with the Laboratory Biosafety Manual, World Health Organization. The macaques were anesthetized with ketamine hydrochloride for inoculation, blood sampling and autopsies.

SHIV infection of macaques

Two pig-tailed macaques (Pt-3944, Pt-3938) were intravenously inoculated with 600 $TCID_{50}$ (corresponding to 1 ng of P27 Gag) of the SHIV-TH09V3 stock propagated in pig-tailed macaque PBMCs. Two cynomolgus macaques (Cy-256, Cy-329) were intravenously inoculated with 10000 $TCID_{50}$ (corresponding to 305 ng of P27 Gag) of the SHIV-TH09V3 stock propagated in cynomolgus macaque splenic mononuclear cells.

Virus isolation from PBMCs and plasma

EDTA-treated whole blood was centrifuged to separate plasma and cellular component. The cellular component was applied for density-gradient centrifugation to purify PBMCs. 5×10^5 of PBMCs or 100 μl of plasma were cocultivated with M8166 cells in 10% FBS-RPMI medium for 4 weeks, with a weekly monitoring of P27 Gag production in the supernatant.



Quantification of plasma viral RNA

Viral RNA (vRNA) was extracted from plasma by QIAamp Viral RNA Kit (Quiagen), followed by real-time RT-PCR carried out and monitored in ABI 7700 PRISM spectrofluorometric thermal cycler (PE Biosystems) as described previously [22]. Each 25-μl reaction mixture contained the followings: 10 μl of the prepared RNA; 1 pmol/μl of each primer (5′AATGCAGAGCCCCAAGAAGAC3′ and 5′GGACCAAGGCCTAAAAAAC CC3′); 0.24 pmol/μl of probe (Fam-5′ACCATGTTATGGCCAAATGCCCAGAC3′-Tamra); 1 × TaqMan™ EZ buffer; 0.3 mM of each dATP, dCTP and dGTP; 0.6 mM of dUTP; 0.25 Unit of AmpErase UNG; 2.5 Units of rTth DNA Polymerase (TaqMan™ EZ RT-PCR Kit, PE Biosystems). RT-PCR condition was as follows; 50 °C for 2 min, 60 °C for 30 min, 95 °C for 5 min, followed by 50 cycles of 95 °C for 5 sec and 62 °C for 30 sec. The control RNA, whose serial dilution served standard curve, was prepared from pKS460 containing SIVmac239 gag under T7 promoter by using MEGAscript™ (Ambion). RNA recovery rate from plasma was determined by doing a parallel purification of the control RNA, as a reference for calculation of plasma vRNA copy number. The limit of detection was approximately 500 RNA copies/ml.

Flow cytometry

50 μl of EDTA-treated peripheral blood was incubated with FITC-conjugated anti-CD3 monoclonal antibody (Mab) (FN-18, Biosource), PE-conjugated anti-CD4 Mab (SK-3, Becton Dickinson) and PerCP-conjugated anti-CD8 Mab (SK-1, Becton Dickinson). Erythrocytes were lysed by FACS Lysing Solution (Becton Dickinson), followed by addition of 50 μl of Flow-count beads solution (Beckman Coulter). CD4- and CD8-positive T-lymphocytes

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Fig. 1. Construction of the pMD14-based V3-chimera SHIV DNAs, and protein profiles of the SHIVs. **A**. Deduced amino acid sequences of V3 loops: subtype E non-syncytium-inducing (NSI) V3 consensus sequence from Thailand [11, 15]; subtype E HIV-1 strains (TH09V3, NH2V3, KH005V3 and NH1V3) substituted for V3-chimera construction; and parental subtype B HIV-1 strain (MD14V3). Underline, N-glycosylation motif at which first N (Asparagine) is potentially glycosylated. *Italic letters*, basic amino acids exchanged with respect to the subtype E NSI V3 consensus sequence. The consensus sequence is completely the same as TH09V3. **B**. Construction scheme. Overlapping primers 2 and 3, and outer primers 1 and 4 were used to generate recombinant DNA segments carrying subtype E V3 and MD14 flanking sequences by overlap extension method [14]. The final PCR products were digested with *Bgl* II and *Bsu*36 I and cloned into the HIV-1 gp120 subclone, pUC-MDH [23]. Subsequently, the *Bgl* II-*Bsu*36 I fragment of the pUC-MDH was cloned into pMD14 to reconstitute a full-length SHIV molecular clone. **C**. Profiles of the viral proteins expressed in HeLa cells transfected with the SHIVs. Cell lysates were separated by 12% polyacrylamide gel and analyzed by Western blot with the following antibodies: anti-SIVmac macaque serum, positive control antiserum of LAV BLOT 1 kit, antiserum of J28 patient [23], and antiserum of NH1 patient [23]. Lanes: *1*, mock transfection; *2*, SHIV-TH09V3; *3*, SHIV-NH2V3; *4*, SHIV-KH005V3; *5*, SHIV-NH1V3; *6*, $SHIV_{MD14}$, respectively. Bands of Gag products (P55 precursor, P27 capside, P17 matrix) and GP160 Env are indicated. P41 band (possible Env transmembrane protein or partially-cleaved Gag product, or both) are also indicated. **D**. Differential V3 immunoreactivity between the SHIVs was further focused by Western blot analysis using two primary antibodies against V3 sequences (RC25 monoclonal antibody and a rabbit antiserum raised against the consensus V3 sequence of NSI HIV-1 E from Thailand). Bands of GP160 Env and GP120 Env are indicated

($CD3^+$) in FSC-SSC lymphocytes-gate were analyzed by FACS Caliber (Becton Dickinson). Absolute counts of the T-lymphocyte subpopulations were determined by referring the known beads count.

Seroconversion analysis

Plasma was incubated at 56 °C for 30 min, and then used as a primary antibody for LAV BLOT 1 Western blot kit.

GHOST cells assay

GHOST cells [2, 30], human osteocarcinoma cells transfected with genes for human CD4 and either human CXCR4 or CCR5, were cultivated in flasks with 10% FBS-DMEM containing 500 μg/ml of gentamycin, 50 μg/ml of hygromycin and 1 μg/ml of puromycin. Trypsin-detached GHOST cells were washed, resuspended with the medium and then seeded into 96-well plates (8×10^2 cells per a well). After an overnight cultivation, culture medium was aspirated out, followed by application of SHIV diluted serially by the medium. After an additional overnight incubation, the applied viral supernatant was aspirated out, and the wells were washed, and then 250 μl of the medium was loaded per a well. After additional 48 hours, culture supernatants were monitored for P27 Gag production.

Results

Construction and preparation of V3-chimera SHIV clones

Four V3-chimera SHIVs were constructed (Fig. 1A and 1B). TH09V3 and NH2V3 are the V3 sequences from non-syncytium-inducing (NSI) HIV-1_{TH09} [11] and HIV-1_{NH2} [23], respectively. KH005V3 and NH1V3 are those of syncytium-inducing (SI) HIV-1_{KH005} [34] and HIV-1_{NH1} [23], respectively.

SHIV stocks were prepared from supernatants of the cultures in which HeLa cells were transfected by the SHIV DNA constructs and then cultivated for 48 to 72 hours. All the viral stocks had RT activity in the range of $1.5 - 2.0 \times 10^3$ cpm/ μl. We named respective V3-chimera SHIVs as SHIV-TH09V3, SHIV-NH2V3, SHIV-KH005V3 and SHIV-NH1V3.

Protein profiles of SHIVs

Western blot analysis of the lysate of the transfected HeLa cells was performed to examine the profiles of viral proteins (Fig. 1C). Anti-SIVmac antiserum recognized SIVmac Gag products: P55 precursor, P27 capside, P17 matrix, and a possible partially-cleaved Gag P41 product. While P27 Gag was mainly detected by the positive control antiserum of LAV BLOT 1 kit, additional GP160 Env band and a 41 kDa band (possible Env transmembrane protein or a partially-cleaved Gag product, or both) were also detected by antiserum of J28 patient [23]. Although antiserum of NH1 patient [23] recognized P27 Gag evenly all through the SHIVs, its recognition of GP160 Env was extensive for SHIV-NH2V3, weak for SHIV-TH09V3, and undetectable for other SHIVs.

Differential V3 immunoreactivity between the SHIVs was further focused by two primary antibodies against V3 sequences (Fig. 1D). GP160 Env and GP120

Env were detected extensively for $SHIV_{MD14}$, but weakly or at the background level for the other SHIVs, by RC25 monoclonal antibody. GP160 Env was detected moderately for SHIV-TH09V3 and $SHIV_{MD14}$, and weakly for SHIV-KH005V3, by a rabbit antiserum raised against the consensus V3 sequence of NSI HIV-1 E from Thailand [11, 15].

In vitro replication of SHIVs in human and macaque PBMCs

The SHIV stocks were evaluated for their replication in human PBMCs by monitoring RT activity (Fig. 2A). $SHIV_{MD14}$ showed the most extensive replication kinetics. SHIV-TH09V3 replicated more slowly, but reached a similar

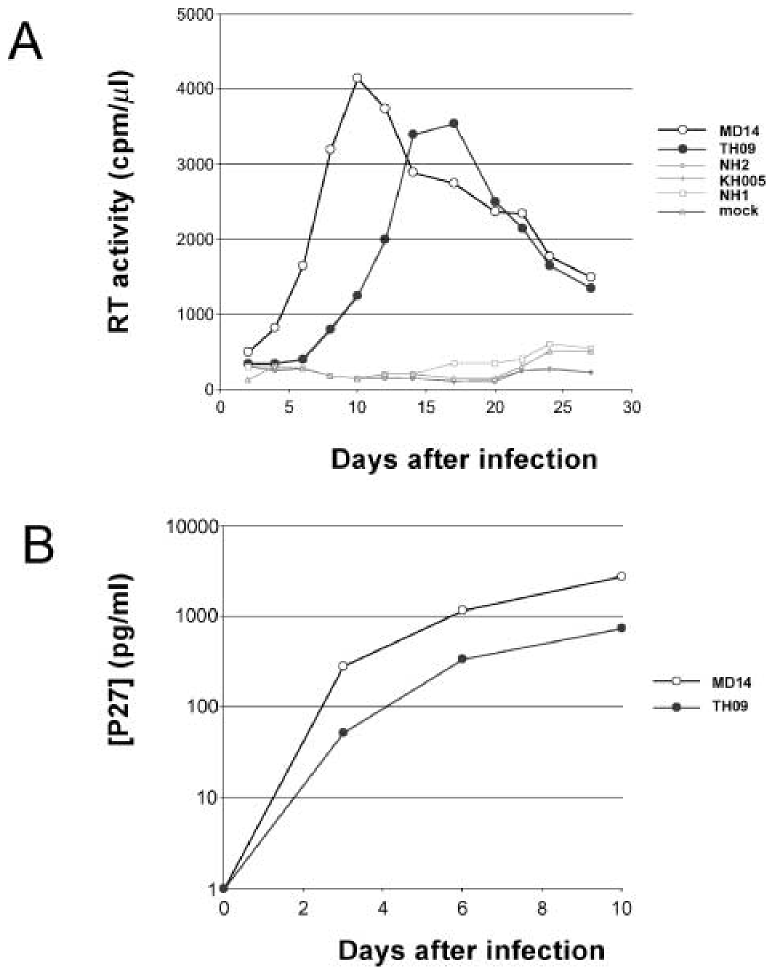

Fig. 2. *In vitro* replication of the SHIV stocks generated by transfecting HeLa cells with the recombinant plasmid DNAs. **A**. PHA-stimulated human PBMCs were infected with the SHIV stocks containing equal amounts of RT activities, followed by RT activity analysis for monitoring SHIVs replication. **B**. ConA-stimulated pig-tailed macaque PBMCs were infected with $SHIV_{MD14}$ and SHIV-TH09V3 stocks, followed by quantifying P27 Gag production

peak height. However, the other three V3-chimera SHIVs did not replicate substantially.

The SHIV stocks were also studied for their replication in PBMCs of two pig-tailed macaques. Only the same two SHIVs, $SHIV_{MD14}$ and SHIV-TH09V3, but no others replicated (data not shown).

Propagation of SHIV-TH09V3 for in vivo *inoculation*

Since only SHIV-TH09V3, out of the four V3-chimera SHIVs, replicated in human and macaque PBMCs, we selected SHIV-TH09V3 for *in vivo* infection of macaques.

For preparation of inoculum to pig-tailed macaques, ConA-stimulated PBMCs of a pig-tailed macaque were infected with the SHIV-TH09V3 stock and cultivated. P27 Gag accumulated up to 744 pg/ml in the culture supernatant on 10th day after infection (Fig. 2B). The virus titer of this inoculum was 600 $TCID_{50}$/ml, as determined by coculture with M8166 cells. Parallel infection with $SHIV_{MD14}$ resulted in 4-fold greater concentration of P27 Gag (Fig. 2B) and in 67-fold greater virus titer.

For preparation of inoculum to cynomolgus macaques, ConA-stimulated splenic mononuclear cells of a cynomolgus macaque were infected with the SHIV-TH09V3 stock. P27 Gag accumulated up to 61 ng/ml (data not shown). The virus titer of this inoculum was 2000 $TCID_{50}$/ml. Parallel $SHIV_{MD14}$ infection resulted in 2.6-fold greater concentration of P27 Gag and in 100-fold greater virus titer.

SHIV-TH09V3 infection of pig-tailed macaques

Two pig-tailed macaques (Pt-3944, Pt-3938) were intravenously inoculated with 800 $TCID_{50}$, corresponding to 1 ng of P27 Gag, of the SHIV-TH09V3 inoculum propagated in pig-tailed macaque PBMCs. For Pt-3944, plasma vRNA load peaked at 2.5×10^7 copies/ml on postinoculational day (PID) 14, followed by a gradual decrease down to 1.2×10^3 copies/ml on PID 40 (Fig. 3A). Pt-3944 showed plasma P27 Gag antigenemia with a peak of about 200 pg/ml on PID 14 (Fig. 3B). Virus was isolated from this macaque PBMCs on PID 11 by coculture with M8166 cells, but not from its plasma. For Pt-3938, plasma vRNA load peaked at 4.5×10^4 copies/ml on PID 10, followed by fluctuations in range between 10^3 to 3×10^4 copies/ml up to PID 25 (Fig. 3A). Pt-3938 showed subtle peak of plasma antigenemia (51 pg/ml) on PID 16 (Fig. 3B). Virus was not isolated from this macaque.

Absolute counts of both CD4- and CD8-positive T-lymphocytes in peripheral blood of these two macaques decreased transiently during primary viremia (Fig. 3C).

Pt-3944 became seroconverted as detected by a conventional Western blot kit (Fig. 3D). P25 Gag protein band became faintly positive on PID 21, and then was apparently positive from PID 28 to PID 40, a day of autopsy. GP160 Env protein

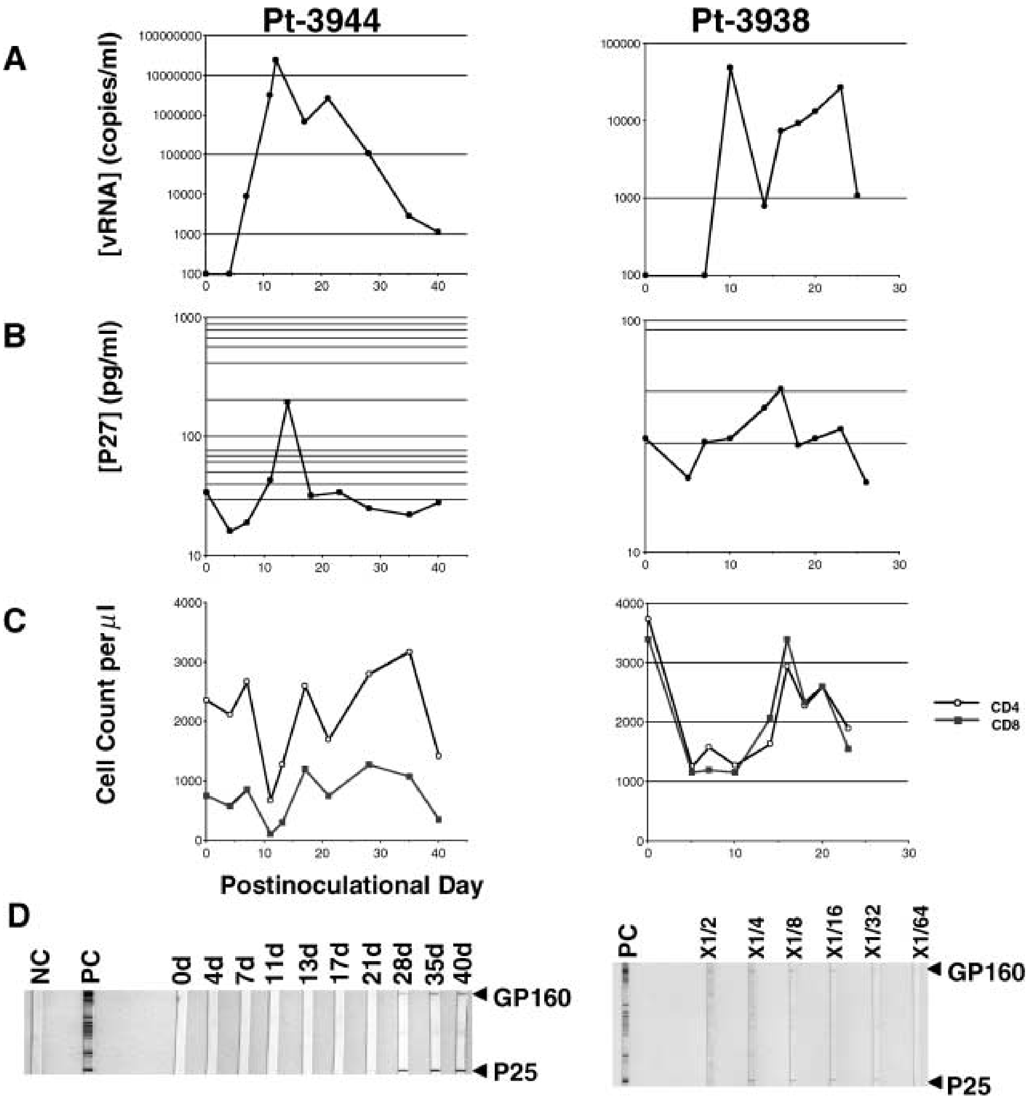

Fig. 3. *In vivo* infection of pig-tailed macaques with SHIV-TH09V3. Two pig-tailed macaques (Pt-3944, Pt-3938) inoculated with SHIV-TH09V3 were monitored for plasma vRNA load (**A**), plasma P27 Gag antigenemia (**B**), absolute counts of CD4- and CD8-positive T-lymphocytes in peripheral blood (**C**) and seroconversion (**D**). For seroconversion analysis, Western blot kit for HIV-1 (LAV BLOT 1) was used. NC and PC, internal negative and positive control of the kit, respectively. Major bands (GP160 and P25) on the strips are indicated. Serial dilutions of PID27-plasma of Pt-3938, as primary antibodies, were applied for confirming the banding specificity. Biologically infectious virus was isolated by coculture with M8166 cells only from PID 11-PBMCs of Pt-3944

band became faintly positive on PID28, and apparent from PID 35 to 40. For Pt-3938, P25 Gag protein band was faintly detectable on PID 23 and 27, an autopsy day. GP160 Env protein band became faintly detectable on PID 27. In order to confirm the specificity of the two faint bands detected for Pt-3938, the plasma of

PID 27 was serially diluted as primary antibodies. Overloading clarified the two bands, consolidating the banding specificity (Fig. 3D).

SHIV-TH09V3 infection of cynomolgus macaques

Two cynomolgus macaques (Cy-256, Cy-329) were intravenously inoculated with 10000 $TCID_{50}$ of the SHIV-TH09V3 inoculum propagated in cynomolgus macaque splenic mononuclear cells. Plasma vRNA load of Cy-256 and Cy-329 peaked on PID 7 at 3.6×10^7 and 4.0×10^6 copies/ml respectively, followed by a continuous decline down to undetectable level on PID 28 (Fig. 4A). Plasma P27 Gag antigenemia for these two macaques was under detectable level thoroughly.

For Cy-256, viruses were isolated from plasma of PID 11, and from PBMCs of PID 14, 18 and 28. For Cy-329, viruses were isolated from plasma of PID 7 and 11, and from PBMCs of PID 11, 14 and 18.

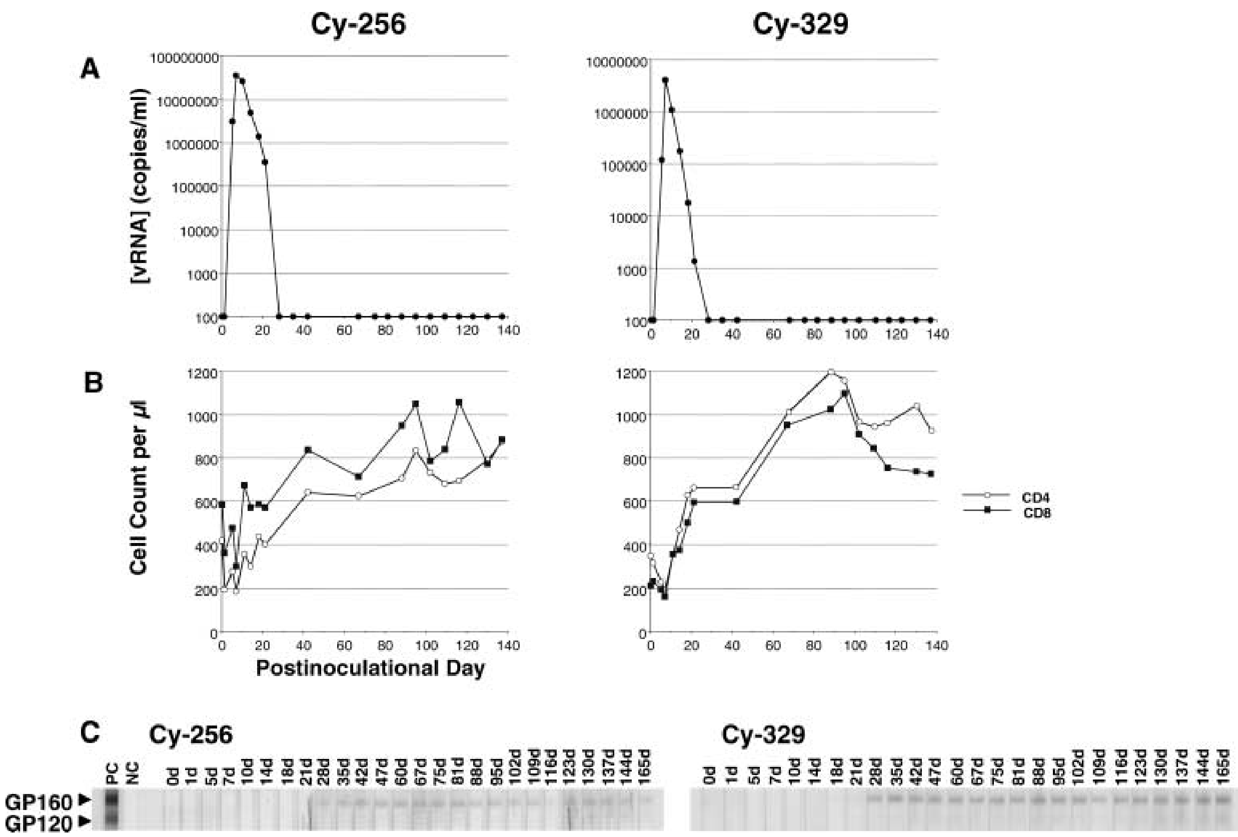

Fig. 4. *In vivo* infection of cynomolgus macaques with SHIV-TH09V3. Two cynomolgus macaques (Cy-256, Cy-329) inoculated with SHIV-TH09V3 were monitored for plasma vRNA load (**A**), absolute counts of CD4- and CD8-positive T-lymphocytes in peripheral blood (**B**) and seroconversion (**C**). Plasma P27 Gag antigenemia was not detected at all the time points monitored. Biologically infectious viruses were isolated from; PID11-plasma and PID 14, 18, 28-PBMCs of Cy-256; PID 7, 11-plasma and PID 11, 14, 18-PBMCs of Cy-329

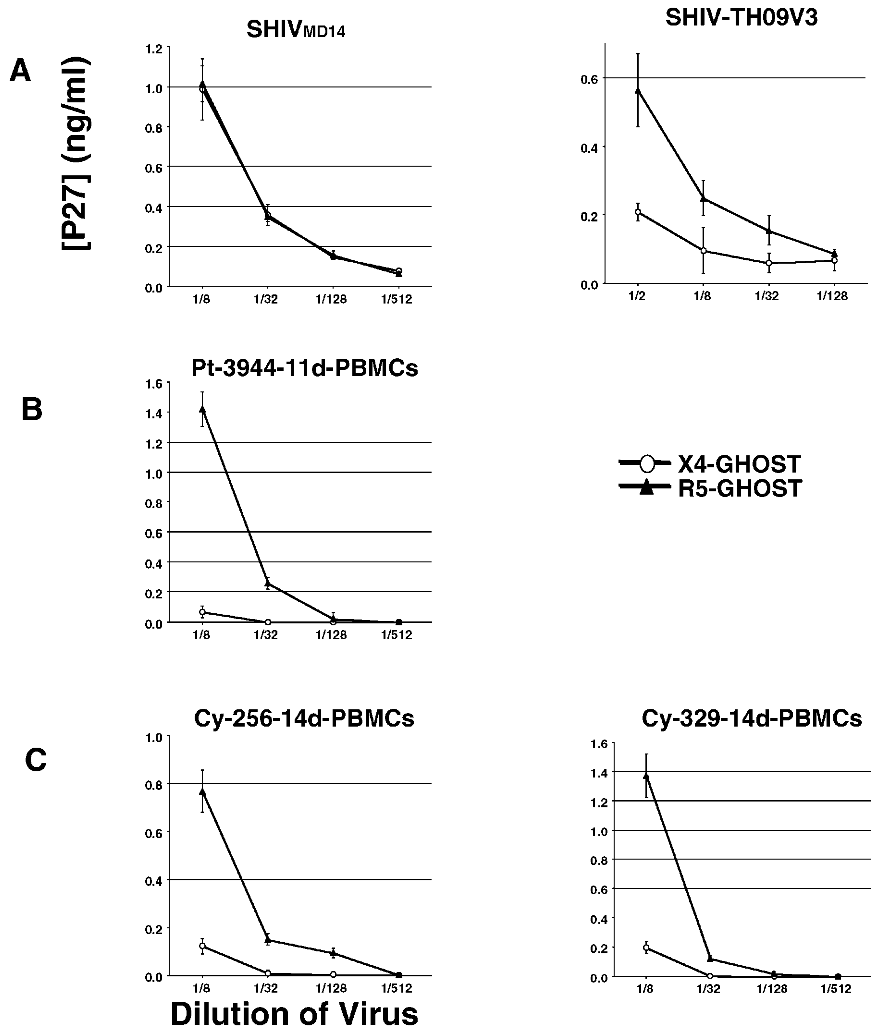

Fig. 5. Coreceptor usage analysis by GHOST cells assay. **A**. $SHIV_{MD14}$ and SHIV-TH09V3 propagated in human PBMCs were verified for their coreceptor usage. **B**. Isolated virus from PID 11-PBMCs of Pt-3944 (infected with SHIV-TH09V3) was analyzed. **C**. Isolated viruses from PID 14-PBMCs of Cy-256 and Cy-329 (both infected with SHIV-TH09V3) were analyzed. Isolated viruses (**B** and **C**) were prepared by coculture with M8166 cells. All the data shown here (**A**, **B** and **C**) are the representatives of three (or more) independent experiments done in triplicate

Absolute counts of both CD4- and CD8-positive T-lymphocytes in peripheral blood of the two macaques showed gradually increasing trends (Fig. 4B).

The two macaques became seroconverted for GP160 Env on PID 28 and thereafter (Fig. 4C).

Coreceptor usage in vitro *and after isolation from macaques*

$SHIV_{MD14}$ and SHIV-TH09V3 were verified for their coreceptor usage by GHOST cells assay [2, 30]. $SHIV_{MD14}$ prepared in human PBMCs replicated equally in

GHOST-X4 and GHOST-R5 cells as measured by P27 Gag production in the culture supernatants. In contrast, SHIV-TH09V3 prepared in the same human PBMCs replicated more extensively in GHOST-R5 cells than GHOST-X4 cells, indicating R5-tropic phenotype (Fig. 5A).

We also verified the coreceptor usage of the isolated viruses from macaques by coculture with M8166 cells. The isolate from PID 11-PBMCs of Pt-3944 showed R5-tropic phenotype (Fig. 5B). The isolates from PID 14-PBMCs of both Cy-256 and Cy-329, were also R5-tropic (Fig. 5C).

Discussion

In this study, we established SHIV-TH09V3, a V3-chimera clone carrying subtype E NSI V3 [11, 15] substituted into $SHIV_{MD14}$ backbone [26] bearing a subtype B envelope. SHIV-TH09V3 replicated in pig-tailed macaque PBMCs and cynomolgus macaque splenic mononuclear cells as well as in human PBMCs. Additionally, SHIV-TH09V3 infected both pig-tailed and cynomolgus macaques. In contrast to X4/R5-dual tropic phenotype of the parental $SHIV_{MD14}$, SHIV-TH09V3 was R5-tropic *in vitro* and after isolation from macaques.

Attempting to construct subtype E V3-chimera SHIV clones, we initially selected the four subtype E V3 sequences (Fig. 1A), which were previously used for constructing subtype E V3-chimera HIV-1 clones with a backbone of subtype B HIV-1 [24]. In that study, all the V3-chimera HIV-1 clones infected human PBMCs and showed the phenotypes conferred from the respective V3 sequences in terms of NSI/SI and X4/R5 coreceptor usage. These results suggested reliability on the strategy of constructing V3-chimera virus clones for evaluating V3-governed phenotypes.

In this study, only SHIV-TH09V3, out of the four V3-chimeras, replicated in human and macaque PBMCs although all the four clones produced equal amount of RT activity in the culture supernatants of the transfected HeLa cells as the parental $SHIV_{MD14}$ did. Further study of profiles of viral proteins produced by these SHIVs in HeLa cells confirmed that Env and Gag proteins were produced evenly all through the SHIVs (Fig. 1C). Additionally, successful V3 replacement was confirmed not only by DNA sequencing but also by differentially specific immunoreactivity of Env protein bands by NH1 patient antiserum (Fig. 1C) and by the two V3-taegeted antibodies (Fig. 1D). Taken together, these results imply that any of the V3 replacements do not prevent production of major structural proteins nor makeup of SHIV virions containing RT activity, but that the combination of V3 and $SHIV_{MD14}$ backbone allowed only SHIV-TH09V3 to replicate in human and macaque PBMCs.

All the pig-tailed and cynomolgus macaques studied here were infected by intravenous inoculation with SHIV-TH09V3. Direct comparison of infectious aspects between the two macaque species is limited because the amount of viral inoculation was more in the cynomolgus macaques than in the pig-tailed macaques. Nevertheless, species differences of infectious aspects can be pointed out. The two cynomolgus macaques allowed virus isolation at several time points from both

PBMCs and plasma. But only one of the two pig-tailed macaques allowed virus isolation at a sole time point only from PBMCs. In contrast, P27 Gag antigenemia in plasma was detected only in the pig-tailed macaques but not in the cynomolgus macaques. As for vRNA viremia after the peak, the cynomolgus macaques showed a continuous decline down to undetectable level, while the pig-tailed macaques rather showed fluctuations after the peak (Fig. 3 and Fig. 4). It remains to be known why this discrepancy, higher antigenemia and more extended post-peak vRNA viremia but lower virus isolation for the pig-tailed macaques than the cynomolgus macaques, occurred. Another point was that absolute counts of both CD4- and CD8-positive T-lymphocytes in peripheral blood of the two cynomolgus macaques showed gradually increasing trends (Fig. 4B). Studying activation markers expressed on these cells may give clues for reasoning this increase.

GHOST cells assay revealed that SHIV-TH09V3 maintained R5-tropic phenotype both *in vitro* and after isolation from macaques. In contrast, $SHIV_{MD14}$ was X4/R5-dual tropic *in vitro* (Fig. 5A). In a preliminary experiment, another cynomolgus macaque was inoculated with $SHIV_{MD14}$ and the isolated viruses were X4/R5-dual tropic (data not shown). These results indicate that the V3 sequences, only the initial difference between SHIV-TH09V3 and $SHIV_{MD14}$, decide the coreceptor usage both *in vitro* and after isolation from macaques. The notion that V3 loops determine coreceptor usage in a relatively independent manner [6, 8, 24, 29, 32] is supported here even in a SHIV backbone.

There are totally 9 amino acids substituted in V3 sequences between SHIV-TH09V3 and $SHIV_{MD14}$ (Fig. 1A). Both SHIVs hold the “R5-usage consensus motif in V3 domain”, 11-S/GXXXGPGXXXXXXXE/D-25, suggested by cross-subtype analysis including subtype E [33]. $SHIV_{MD14}$ bears a whole envelope of HIV-1_{DH12}, except minor differences at the both ends [26]. HIV-1_{DH12} is X4/R5-dual tropic, dictating its coreceptor usage phenotype to $SHIV_{MD14}$ [27]. Recently, N-glycosylation site in HIV-1_{DH12} V3 was suggested to be critical for R5-usage ability [20]. The N-glycosylation motif in V3 (Fig. 1A) is also conserved in SHIV-TH09V3, being consistent with the maintained R5 usage.

So far, two SHIV clones carrying an envelope of subtype E HIV-1 have been reported. $SHIV_{9466.33}$ [16], bearing an envelope of SI T cell tropic clinical isolate from Thailand, infected baboons. Since SI T cell tropic phenotype is mostly accompanied with X4-tropic phenotype [12], $SHIV_{9466.33}$ can be predicted either X4-tropic or X4/R5-dual tropic. SHIV-E-CAR [13], bearing an envelope of HIV-1 clinical isolate from Central African Republic, infected rhesus macaques progressively becoming pathogenic after animal-to-animal passages while maintaining X4-tropic phenotype. Compared with the two non-R5-tropic SHIVs, SHIV-TH09V3 may serve as an R5-tropic subtype E HIV-1 model in a V3-targeted study. We are also pleased that SHIV-TH09V3 carries the consensus V3 sequence of subtype E NSI HIV-1 from Thailand [11, 15], reasoning itself as a representative virus model. Additionally, infection of macaques with SHIV-TH09V3 and $SHIV_{MD14}$ will serve a unique animal model for focusing on difference of the outcome caused by the different V3 sequences in connection with coreceptor usage.

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Author’s address: Dr. Mitsuo Honda, AIDS Research Center, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan; e-mail: mhonda@nih.go.jp