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Species Tropism of Chimeric SHIV Clones Containing HIV-1 Subtype-A and Subtype-E Envelope Genes

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To analyze HIV-1 genes in a nonhuman primate model for lentivirus infection and AIDS, recombinant SIV/HIV-1 (SHIV) clones were constructed from two HIV-1 subtype-A isolates (HIV-1_{SF170} and HIV-1_{Q23-17} from individuals in Africa) and two HIV-1 subtype-E isolates (HIV-1_{9466} and HIV-1_{CAR402} from AIDS patients in Thailand and Africa), respectively. These four SHIV clones, designated SHIV-A-170, SHIV-A-Q23, SHIV-9466.33, and SHIV-E-CAR, contain envelope (*env*) genes from the subtype-A or -E viruses. Interestingly, SHIV-A-170, SHIV-A-Q23, and SHIV-9466.33 were restricted for replication in cultures of macaque lymphoid cells, whereas SHIV-E-CAR replicated efficiently in these cells. Additional studies to define the block to replication in macaque cells were focused on the subtype-E clone SHIV-9466.33. A SHIV intragenic *env* clone, containing sequence-encompassing V1/V2 regions of HIV-1_{CAR402} and V3/V4/V5 regions of SHIV-9466.33, infected and replicated in macaque lymphoid cells. These results indicated that the sequence-encompassing V1/V2 region of HIV-1_{9466} was responsible for the block of the SHIV-9466.33 replication in macaque cells. Analysis of viral DNA in acutely infected macaque cells revealed that SHIV-9466.33 was blocked at a step at/or before viral DNA synthesis, presumably during the process of virion entry into cells. In a fluorescence-based cell–cell fusion assay, fusion pore formation readily took place in cocultures of cells expressing the SHIV-9466.33 *env* glycoprotein with macaque T-lymphoid cells. Taken together, these results demonstrated that the block of SHIV-9466.33 replication in macaque cells is at an early step after fusion pore formation but before reverse transcription.

INTRODUCTION

Genetic variability is the hallmark of human immunodeficiency virus type 1 (HIV-1). Diverse HIV-1 genotypes, identified in the worldwide epidemic, have been classified into genetic subtypes (A through K) in group M, as well as the highly divergent groups O and N (Robertson *et al.,* 2000). The *env* glycoprotein of HIV-1 governs several viral properties (i.e., cell tropism, cytopathology, and coreceptor usage) and is the major target for antiviral immune responses. Thus, the high degree of sequence variability of HIV-1 *env* presents a significant challenge for vaccine development. HIV-1 subtype-A is prevalent in Africa, which contains the majority of people living with HIV-1 and AIDS in the world (Essex, 1999). HIV-1 subtype-E, identified as a unique subtype by genetic sequences in *env*, was first reported in Thailand (McCutchan *et al.,* 1992; Ou *et al.,* 1992); this subtype has also been found in Africa (Murphy *et al.,* 1993; Nkengasong *et al.,* 1994). At present, subtype-E viruses are the most prevalent strains in Thailand and neighboring nations in Asia (Brown and McNeil, 1998). Subtype-E viruses have also entered other continents (Artenstein *et al.,* 1995; Böni *et al.,* 1999; Brodine *et al.,* 1995; Couturier *et al.,* 2000). The global epidemic of these and other viral subtypes will impact the development and production of HIV-1 vaccines (Brown and McNeil, 1998; Essex, 1999). Additionally analysis of phenotypes of different HIV-1 subtypes may be important for understanding viral transmission and pathogenesis (Hu *et al.,* 1999)

The lack of an animal model for HIV-1 infection and fatal immunodeficiency has hindered the elucidation of determinants of transmission and pathogenesis of different viral subtypes. Chimeric SHIV containing the *env* gene of several HIV-1 subtype-B strains and one HIV-1 subtype-C strain replicated efficiently *in vitro* in macaque cell cultures and established either transient or persistent infection in macaques (Chen *et al.,* 2000; Joag, 2000; Nath *et al.,* 2000). SHIV strains that infect, persist, and cause immunodeficiency in macaques are important challenge viruses for evaluating efficacy of vaccines based on HIV-1 *env* glycoprotein immunogens. Our previous studies demonstrated that the chimeric SHIV-E-CAR clone, containing the *env* gene of HIV-1_{CAR402} (subtype-E virus from the Central African Republic), readily infected rhesus macaques through intravenous or intravaginal inoculations (Himathongkham *et al.,* 2000). In contrast, another subtype-E chimera SHIV-9466.33, containing the *env* gene of HIV-1_{9466} (subtype-E virus from Thailand), was restricted for replication in macaque lymphoid cells (Klinger *et al.,* 1998). In the current article, we

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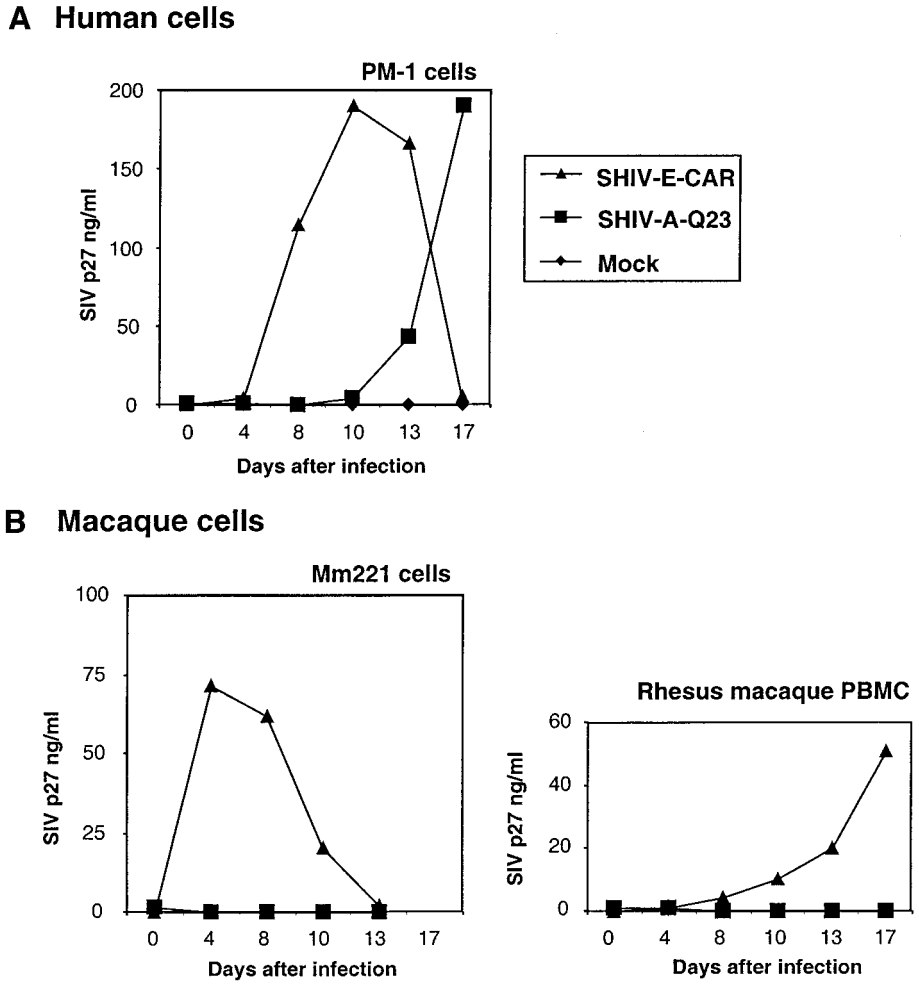


FIG. 1. Replication of subtype-A SHIV-A-Q23 clone in human and macaque cells. SHIV-A-Q23 viral production, measured by ELISA for SIV p27 antigen, was analyzed in (A) human cells (PM-1) and (B) macaque cells (Mm221 and macaque PBMC) at 0, 4, 8, 10, 13, and 17 days postinfection.

also investigated the species tropism of two subtype-A chimeric viruses, SHIV-A-170 and SHIV-A-Q23, containing the *env* gene of HIV-1_{SF170} and HIV-1_{Q23-17} (subtype-A viruses from Rwanda and Kenya, respectively). Both chimeric viruses with subtype-A *env* genes were restricted for replication in macaque cells. Detailed analysis of the species restriction was performed on subtype-E chimeric SHIV-9466.33; our studies revealed that replication was blocked in macaque-T-lymphocyte cultures at an early step, after fusion pore formation but before viral DNA synthesis. Analysis of SHIV clones with the subtype-A or subtype-E *env* genes described here *in vitro* and in nonhuman primates can provide new insight on the mechanism(s) of early events in viral replication.

RESULTS

Comparison of SHIV clones in humans and macaque cells

To analyze HIV-1 subtype-A and subtype-E *env*-gene functions, we compared SHIV clones constructed from four divergent viruses. Selected for subtype-A viruses were HIV-1_{SF170}, a macrophage-tropic virus from an individual in Rwanda, and HIV-1_{Q23-17}, a non-syncytium-inducing isolate from a nonsymptomatic individual in Kenya (Cheng-Mayer *et al.,* 1988; Poss *et al.,* 1998). Additionally, subtype-E viruses chosen for this study were HIV-1_{9466}, a syncytium-inducing isolate from an AIDS patient in Thailand (Ichimura *et al.,* 1994), and HIV-1_{CAR402}, a syncytium-inducing virus isolate from an AIDS patient in the Central African Republic (Gao *et al.,* 1996). All four chimeric clones (SHIV-A-170, SHIV-A-Q23, SHIV-9466.33, and SHIV-E-CAR) produced infectious virus after transfection of 293T cells; infectivity was assessed by coculture of transfected cells with human PBMC.

Replication properties of SHIV subtype-A and subtype-E clones were analyzed by infection of both human and macaque PBMC cultures. The subtype-A chimeric viruses, SHIV-A-Q23 (Fig. 1) and SHIV-A-170 (data not shown), replicated in human lymphoid cells but not in either macaque PBMC or Mm221 cell line. Both subtype-E SHIV clones, SHIV-E-CAR and SHIV-9466.33, rep-

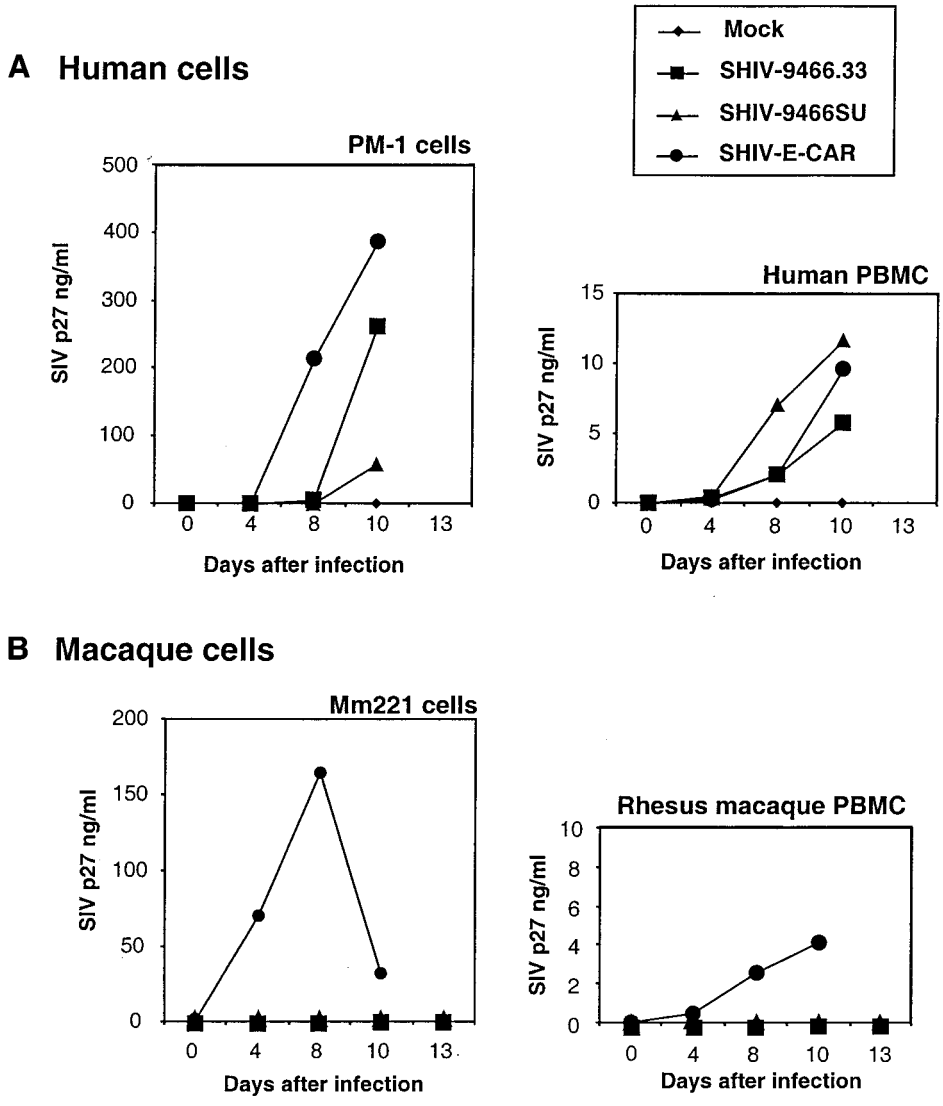


FIG. 2. Replication of SHIV-9466su in human and macaque cells. SHIV-9466.33, SHIV-9466su, and SHIV-E-CAR viral production, measured by ELISA for SIV p27 antigen, was analyzed in (A) human cells (PM-1 and human PBMC) and (B) macaque cells (Mm221 and macaque PBMC) at 0, 4, 8, 10, and 13 days postinfection.

licated efficiently in human lymphoid cells; however, SHIV-9466.33 replication in macaque lymphoid cells was restricted (Fig. 2). Further studies to define region of *env* that governed this species tropism were focused on the subtype-E SHIV-9466.33 clone.

To determine whether *env* gp120 or gp41 of HIV-1_{9466} controls restriction of SHIV-9466.33 in macaque cells, replication of SHIV-9466su was analyzed in human and macaque cells (Fig. 2). SHIV-9466.33, SHIV-9466su, and SHIV-E-CAR replicated efficiently in human PBMC cultures and in PM-1 cells (Fig. 2A). In contrast, both SHIV-9466su and SHIV-9466.33 did not yield detectable virus in either primary macaque cells or the Mm221 line, whereas SHIV-E-CAR replicated efficiently in these cells (Fig. 2B). These results indicated that gp120 of the HIV-1_{9466} (subtype-E) isolate played a critical role in restricting replication of the SHIV clones containing subtype-E *env* sequences of HIV-1_{9466} in macaque cells.

To identify the region(s) in gp120 of the HIV-1_{9466} responsible for restricted replication in macaque cells, intragenic *env* recombinant was constructed from *env* of HIV-1_{9466} and HIV-1_{CAR402}. The first SHIV clone containing an intragenic *env* gene, SHIV-E-R1, was not infectious. The reciprocal recombinant clone, SHIV-E-R2 clone, replicated efficiently in human PBMC and PM-1 cells (Fig. 3A). Interestingly, the SHIV-E-R2 readily infected both Mm221 line and macaque PBMC (Fig. 3B). These findings suggested that the sequence encompassing V1/V2 region of HIV-1_{9466} was responsible for the restriction of SHIV-9466.33 replication in macaque cells.

Analysis of the block to replication of SHIV-9466.33 in macaque cells

Viral DNA synthesis. To determine whether the block to replication was in the early or late phase of the viral

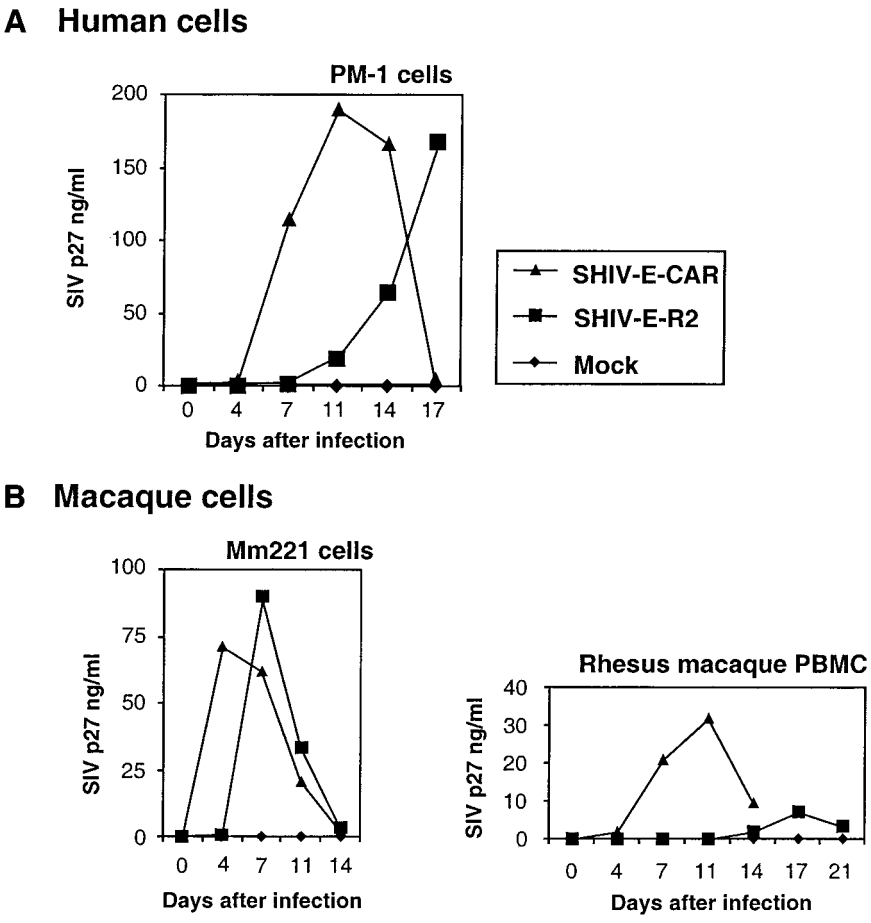


FIG. 3. SHIV-R2 replication in human and macaque cells. SHIV-E-R2 and SHIV-E-CAR viral production, measured by ELISA for SIV p27 antigen, was analyzed in (A) human cells (PM-1) and (B) macaque cells (Mm221 and macaque PBMC) at 0, 4, 7, 11, 14, 17, and 21 days postinfection.

life cycle, the level of viral DNA was analyzed in macaque cells inoculated with SHIV-9466.33. After inoculation of macaque PBMC cultures with a cell-free preparation of SHIV-9466.33, whole-cell DNA was isolated at 0, 6, 12, and 24 h after infection, and viral DNA levels were determined in a semiquantitative PCR amplification system. Complete proviral DNA and strong stop viral DNA were monitored by measuring the amount of the 475 or 295 bp DNA, respectively, produced by PCR amplification with primers specific for SIV sequences (Figs. 4A and 4C). After infection with SHIV-9466.33, viral DNA was barely detected in macaque PBMC (Figs. 4B and 4D). SHIV-9466.33-infected human PBMC and SHIV-SF33-infected macaque PBMC served as positive controls for detection of synthesized viral DNA. Analysis of viral DNA in cultures treated with the reverse transcriptase inhibitor azidothymidine (AZT) was performed to ensure that viral DNA detected in these studies was newly synthesized and not carried over in virions. Taken together, these results demonstrated that the block to SHIV-9466.33 replication in rhesus macaque PBMC was at the early phase of viral infection, involving a step(s) at/or prior to reverse transcription.

Fusion. To examine further whether the block of SHIV-9466.33 infection in macaque lymphocytes took place at the step of fusion entry, we adopted a fluorescent dye transfer system to detect cell–cell fusion mediated by the *env* glycoprotein. This system, described under Materials and Methods, is based on redistribution of cytoplasmic fluorescent dyes between *env*-expressing 293T cells and macaque $CD4^+$ T-lymphoid cells (Mm221 cells). Expression of 9466.33 *env* on transfected 293T cells was detected by immunofluorescence microscopy using monoclonal antibody to HIV-1 gp120 (data not shown). *Env*-expressing 293T cells labeled with the CMTMR dye had high viability (96%) and exhibited an evenly distributed diffuse red fluorescence. Mm221 cells labeled with the CMFDA dye also showed high viability (98%) and a bright green fluorescence. Figures 5A–5C, 5D–5F, and 5G–5I contain panels of images (bright field, red fluorescence, and green fluorescence) obtained 8 h after coculturing CMFDA-labeled macaque $CD4^+$ T-lymphocyte Mm221 cells and CMTMR-labeled 293T cells expressing the *env* gene of HIV-1_{CAR402}, SHIV-9466.33, or SHIV-E-R2, respectively. The lymphocytes adhered to the *env*-expressing 293T cells and many retained a typical spheri-

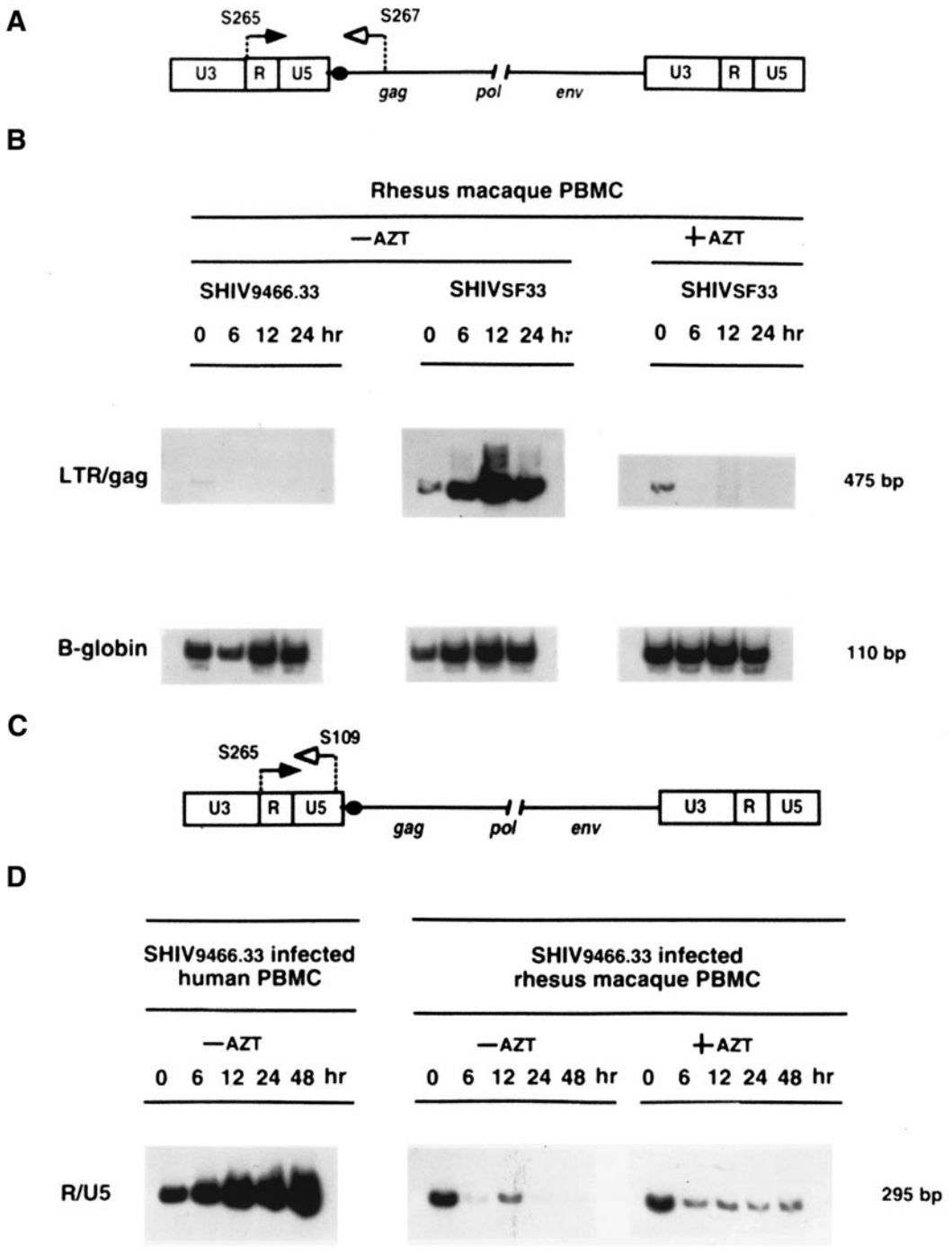
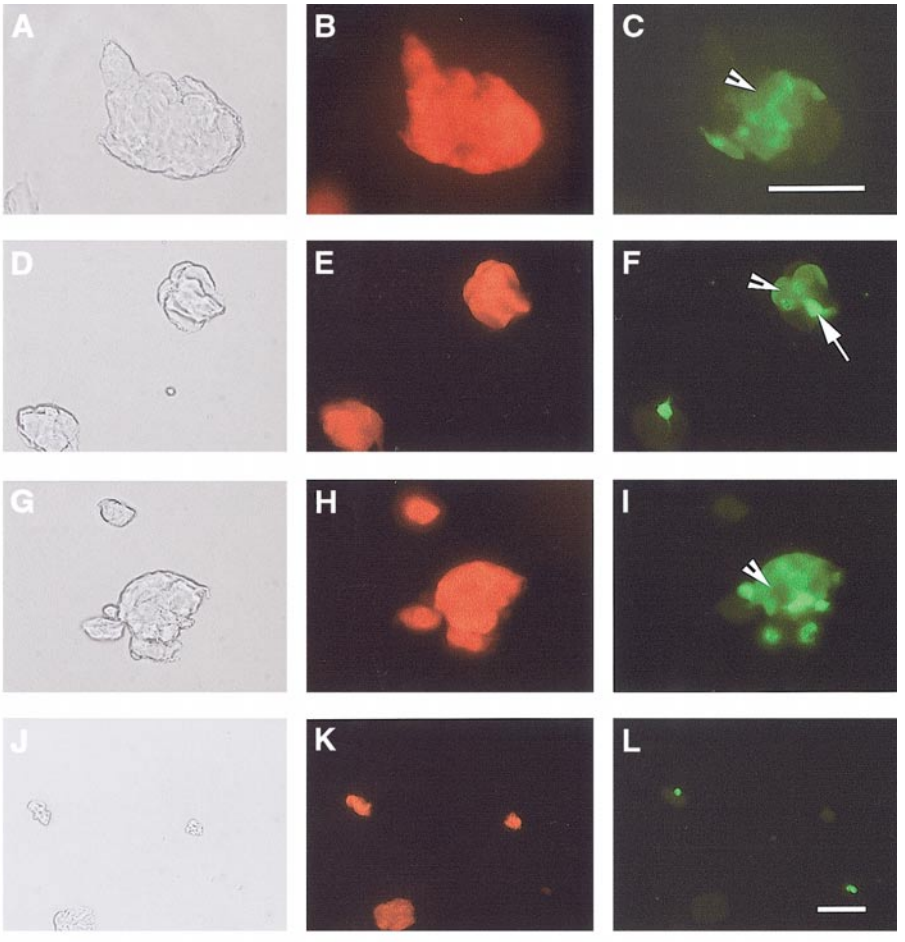


FIG. 4. PCR analysis of SHIV-9466.33 DNA in rhesus macaque PBMC. (A and C) The PCR primer pairs (^{32}P-labeled) specific for LTR/*gag* region of SIV_{mac239}, S265, and S267, and the R/U5 region, S265 and S109, detect a late (475 bp) and an early (295 bp) reverse transcription product. (B and D) Viral DNA was measured in SHIV-9466.33-infected macaque PBMC, in the absence or presence of 10 μM AZT, at 0, 6, 12, and 24 h after infection. SHIV-9466.33-infected human PBMC and SHIV-SF33-infected macaque PBMC served as positive controls. A primer pair specific for the human β-globin was included in all reactions to serve as control. After PCR amplification, DNA products were analyzed by electrophoresis on polyacrylamide gels and visualized by autoradiography of dried gels.

cal morphology with a discreet, bright green fluorescence. When viewed under red fluorescence, these cells frequently appeared as dark "shadows" on the underlying red 293T cells. The white arrow indicates an example of such a cell. Other lymphocytes in these cocultures did not retain their rounded morphology and the green fluorescence appeared to have spread, forming larger areas of diffuse green fluorescence. These areas of diffuse green fluorescence were only seen in cell clusters that consisted of both macaque lymphocytes and 293T cells.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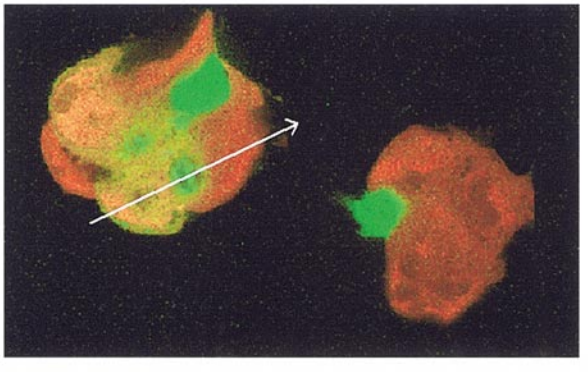

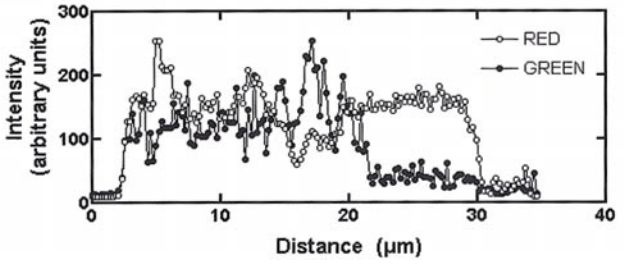
Intensity (arbitrary units)
300
200
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20
30
40
Distance (μm)
RED
GREEN

6

The diffuse green fluorescence frequently colocalized with the red fluorescence associated with the 293T cells. Arrowheads in Fig. 5 indicate examples of areas where there appears to be colocalization of green and red fluorescence. Panels J–L in Fig. 5 show controls, including coculture of macaque lymphocytes incubated with mock-transfected 293T cells. In these control cocultures, the CMFDA-labeled lymphocytes maintained a uniform, rounded morphology and showed little adherence to the 293T cells. Larger areas of diffuse green fluorescence that colocalized with the red fluorescence were not detected.

This fluorescence microscopy procedure to measure *env*-mediated dye-redistribution was used for rapid screening of large numbers of samples and suggested that fusion pore formation was taking place in the *env*-expressing cocultures but not in the control (mock-transfected) coculture. To ensure that the colocalization of green and red fluorescence was due to fusion process and was not due to overlap of cells, selected cocultures were also examined by confocal microscopy. Figure 6 shows one optical section (from a stack of 40) obtained from analysis of a typical CMFDA-labeled Mm221 cell/CMTMR-labeled 9466.33*env*-expressing 293T cell coculture; two cell clusters are apparent. The fluorescence intensity profile corresponding to the region along the white arrow, drawn on the leftmost cell cluster, is also shown in Fig. 6. For this cell cluster, colocalization of both green and red fluorescence, consistent with cell–cell fusion, is clearly observed. Similar evidence of green and red fluorescence colocalization was found upon examining other *env*-expressing 293T cells in this same experiment (data not shown). Confocal imaging of the cell cluster on the right in Fig. 6 showed no evidence of green/red fluorescence colocalization despite the fact that the CMFDA-labeled cell is adherent to the CMTMR-labeled cell colony. This observation provides an internal control that argues against the idea that colocalization of the fluorescent dyes results from dye leakage and dye re-uptake. These results indicate that SHIV-9466.33 replication was restricted in macaque cells at an early step, after fusion pore formation of virion and cell surface membranes.

TABLE 1

Summary of Human and Macaque Tropisms of SHIV Containing Envelope Genes of HIV-1 Subtype-A and Subtype-E Isolates

HIV-1 *env* subtypes	SHIV clones	Replication in human cells		Replication in macaque cells	
		PM-1	PBMC	Mm221	PBMC
B	SHIV-SF33	+	+	+	+
E	SHIV-9466.33	+	+	−	−
E	SHIV-9466su	+	+	−	−
E	SHIV-E-CAR	+	+	+	+
E	SHIV-E-R2	+	+	+	+
A	SHIV-A-170	+	+	−	−
A	SHIV-A-Q23	+	+	−	−

DISCUSSION

Studies on several HIV-1 subtype-B isolates showed that the *env* glycoprotein of this virus plays a major role in virion entry of $CD4^+$ T-lymphocytes and influences cell tropism (Luciw, 1996; Weiss, 1994). Previous work demonstrated that the *env* glycoprotein of HIV-1 subtype-B isolates from North America and Europe is not responsible for the block to infection of macaque cells (Himathongkham and Luciw, 1996; Shibata *et al.,* 1995). Here, we demonstrated that the *env* gene of $HIV\text{-}1_{9466}$, a subtype-E virus from Thailand, was a major factor that restricted replication in macaque cells. Two additional chimeric clones, SHIV-A-170 and SHIV-A-Q23 containing different HIV-1 subtype-A *env* genes, were also restricted in macaque lymphoid cells (Table 1). An R5-tropic chimeric virus, $SHIV_{CHN19}$, containing a subtype-C *env* gene from $HIV\text{-}1_{CHN}$ from China, did not replicate *in vitro* in rhesus macaque lymphocytes but replicated in lymphocytes from pig-tailed macaques (Chen *et al.,* 2000). It remains to be determined whether the subtype-A and subtype-E SHIV clones infect and replicate in pig-tailed macaque cells.

Biochemical analysis revealed that an early step(s) prior to viral DNA synthesis was blocked in replication of the SHIV-9466.33 clone in macaque cells. The colocalization of fluorescent dyes that was observed by fluorescence microscopy and confocal microscopy when 293T

FIG. 5. Analysis of fluorescence dye-redistribution in cocultures of *env* expressing 293T cells and Mm221 cells. Envelope-expressing 293T cells were labeled with 2 μM CMTMR dye and lymphocytic Mm221 cells were labeled with 2 μM CMFDA dye as described under Materials and Methods. The cells were cocultured for 8 h and then examined in a Nikon Eclipse E800 epifluorescence microscope equipped with a CCD camera. For each field of view, images corresponding to bright field, CMTMR dye (red fluorescence), and CMFDA dye (green fluorescence) were captured directly into Photoshop. (A–C) CAR-*env*; (D–F) 9466.33-*env*; (G–I) R2-*env*; (J–L) mock-transfected 293T cells. The arrow indicates a lymphocyte that shows no redistribution of fluorescence. Arrowheads point to examples where green and red fluorescence appears to colocalize. The horizontal lines indicate 50-μm markers. The images in A–I were obtained with a 40× objective and that in J–L with a 20× objective. These experiments were performed at least three times with similar results.

FIG. 6. Analysis of fluorescence dye colocalization by confocal microscopy. 293T cells expressing *env* glycoprotein of SHIV-9466.33 were labeled with 2 μM CMTMR dye (red fluorescence) and macaque lymphocytic Mm221 cells were labeled with 2 μM CMFDA dye (green fluorescence) as described under Materials and Methods. The cells were cocultured for 8 h and then examined by confocal microscopy to obtain a stack of 0.9-μm optical sections. The photomicrograph shows a representative optical section. The graph shows the green/red fluorescence intensities along the white arrow drawn on the micrograph. Similar results were found using 293T cells expressing other HIV-1 *env* glycoproteins.

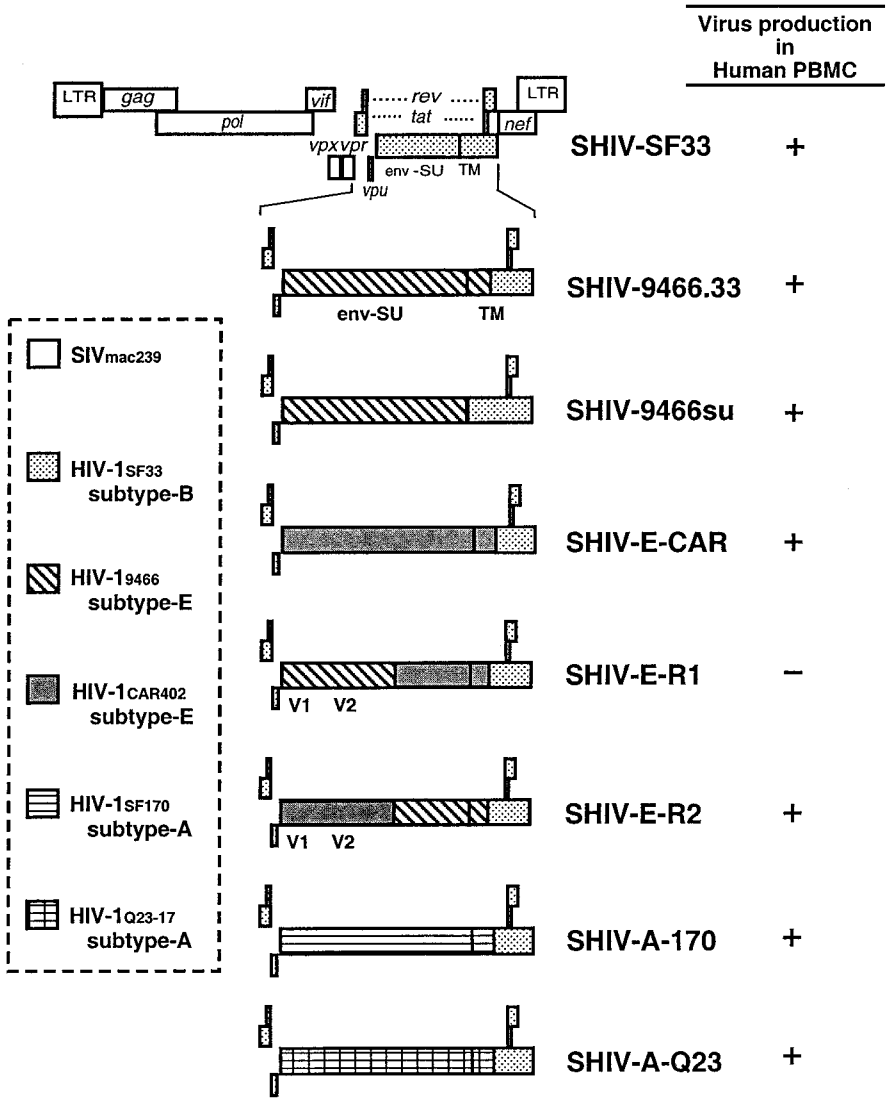

FIG. 7. Structures and viral recovery in human PBMC of chimeric SHIV clones containing *env* region sequences from HIV-1 subtype-A or subtype-E isolates. The SHIV-SF33 chimera is shown at the top. Shaded boxes represent sequences derived from the HIV-1_{SF33} (subtype-B) clone. Boxes with diagonal lines represent sequences derived from the HIV-1_{9466} (Thai subtype-E) isolate; solid gray boxes represent sequences derived from the HIV-1_{CAR402} (African subtype-E) isolate. Boxes with horizontal lines represent sequences derived from HIV-1_{SF170} (Rwanda subtype-A); boxes with grid lines represent sequences derived from the HIV-1_{Q23-17} (Kenya subtype-A). The surface (SU) gp120 and transmembrane (TM) gp41 regions of the envelope glycoproteins are shown.

(human) cells expressing *env* gene of SHIV-9466.33 were cocultured with macaque lymphoid cells is consistent with cell–cell fusion pore formation. Taken together, these findings point to a restriction of SHIV-9466.33 at a step between fusion entry and initiation of viral DNA synthesis.

Experiments were conducted to determine whether a specific domain in the *env* glycoprotein of subtype-E HIV-1_{9466} controlled this restricted tropism. Several *env* intragenic recombinants were constructed between the gp120 region of the *env* genes of HIV-1_{9466} and HIV-1_{CAR402}. Most of the SHIV proviral clones with these intragenic recombinant *env* genes were not infectious in human lymphoid cells (Fig. 7 and data not shown). However, one chimeric proviral clone, SHIV-E-R2, was infectious in both human and macaque lymphoid cells. This finding indicated that the region encompassing the V1/V2 region of HIV-1_{CAR402}*env* was important for allowing replication in macaque cells. The V1/V2 region of the *env* glycoprotein influences coreceptor interaction as well as cell tropism, cytopathicity, and sensitivity to neutralizing antibodies (Hoffman and Doms, 1999). Our results indicated that the V1/V2 region plays an important role in virion entry. In the model proposed above, it is possible that the V1/V2 region of HIV-1_{CAR402}*env* gp120 binds a putative cellular protein, which is essential for infection of macaque cells at the uncoating step in viral replication.

SHIV clones constructed from *env* genes of HIV-1 subtype-B have proven useful for assessing viral deter-

minants of pathogenesis and transmission (Buch *et al.,* 2000; Karlsson *et al.,* 1997; Lu *et al.,* 1998; Shibata *et al.,* 1997). Our article indicates that further studies will be necessary to reliably construct SHIV clones, containing *env* genes of non-subtype-B HIV-1 isolates that replicate in macaque cells. The availability of nonhuman primate model susceptible to infection with the subtype-E SHIV chimeric virus will facilitate the development of HIV-1 vaccines targeting for Thailand and neighboring countries in Asia.

MATERIALS AND METHODS

Construction of SHIV chimera and *env* expression vectors

The cloning strategy for constructing pSHIV-SF33, pSHIV-9466.33, and pSHIV-E-CAR chimeras was described elsewhere (Himathongkham *et al.,* 2000; Klinger *et al.,* 1998; Luciw *et al.,* 1999). Clones pSHIV-A-170 and pSHIV-A-Q23 were also constructed by using the same strategy as mentioned above. Plasmid pSHIV-9466su was constructed by replacing gp41 of pSHIV-9466.33 with the counterpart region of HIV-1_{SF33}. Overlapping PCR amplification was used to generate sequences at the junction of HIV-1_{9466} gp120/HIV-1_{SF33} gp41 from *Mun*I site to *Avr*II site (343 bp). Two PCR primer pairs were designed as shown. Primer SU1: 5′GGAGGAAATATAAAGGACAATTGGAGAAGTG3′, primer SU2: 5′TATCACTCCCACTGCTCTTTTTTCTCTCTGCACCACTCTTCTC3′, primer SU3: 5′ AGAGAGAAAAAAGAGCAGTGGGAGTGATAGGAGCTATGTTCC 3′, primer SU4: CCCCAAATCCCTAGGAGCTGTTGATCTCTTAGGTATC 3′. Underlined nucleotide residues are HIV-1_{9466} sequences. The first primer pair (SU1 and SU2) and the second pair (SU3 and SU4) were used to amplify 115 and 267 bp DNA fragments from HIV-1_{9466} and HIV-1_{SF33}, respectively. PCR products were pooled and used as target for the next PCR amplification by using primers SU1 and SU4 to generate a 343-bp DNA fragment. The DNA fragment was digested with *Mun*I and *Avr*II and then cloned into pSHIV-9466.33 to obtain pSHIV-9466su. Construction of pSHIV-E-R1 and pSHIV-E-R2 was based on standard cloning methods using unique restriction sites (*Bsa*BI and *Xho*I) available in pSHIV-9466.33 and pSHIV-E-CAR. For pSHIV-E-R1, we replaced the DNA fragment encompassing V3V4V5 regions of gp120 and gp41 of pSHIV-E-CAR with the counterpart region of pSHIV-9466.33. Plasmid pSHIV-E-R2 was constructed as a reciprocal clone of pSHIV-E-R1 (Fig. 7). All constructs were verified by DNA sequencing. Accession number for the *env* gene of HIV-1_{SF170}, kindly provided by C. Cheng-Mayer (Aaron Diamond Center, NY), is M66533; accession number for HIV-1_{Q23-17}, kindly provide by J. Overbaugh (University of Washington, WA), is AF004885.

The gp160 *env* genes of SHIV-E-CAR, SHIV-9466.33, and SHIV-E-R2 were amplified by PCR and individually cloned into the *env* expression plasmid, pND14, which contains the cytomegalovirus immediate early gene promoter (Luciw, 1999). Plasmids were transfected into 293T cells and the time for optimal expression was determined by immunofluorescence microscopy and Western blot analyses of the transfected cells. Mouse monoclonal anti-HIV-1 gp120 antibody was generously provided by P. L. Earl, NIAID, NIH.

Cell culture

The human embryonic kidney 293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine. PM-1 cells, a human T-cell line, was obtained from Robert Gallo (University of Maryland, MD) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 u/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine. The macaque T cell line Mm221 was obtained from Ron Desrosiers (New England Primate Research Center, Southboro, MA) (Alexander *et al.,* 1997) and cultured in RPMI 1640 medium supplemented with 20% fetal calf serum and recombinant human interleukin-2 (IL-2) at 75 u/ml (AIDS Research and Reference Reagent Program, NIH, Bethesda, MD; Chiron Corp., Emeryville, CA), 2 β-mercaptoethanol, and 25 mM HEPES buffer. Culture medium for human and rhesus macaque PBMC was complete RPMI 1640 medium supplemented with recombinant human IL-2 at 50 u/ml. Human PBMC from a healthy, HIV seronegative donor were prepared by Ficoll–Hypaque density centrifugation of heparinized blood resuspended in the culture medium described above and stimulated with 5 μg/ml phytohemagglutinin for 3 days. PBMC from rhesus macaques were also prepared by the same procedure except these cells were stimulated with 0.5 μg/ml *Staphylococcus* enterotoxin A (Toxin Technology, Inc., Sarasota, FL).

Transfection of cloned proviral DNA to recover virus

To recover virus from cloned DNA plasmids, 10 μg pVP-1, containing the 5′ half of the pathogenic clone SIV_{mac239}, was digested with *Sph*I and ligated to 10 μg of the 3′ half of the SHIV genome, which also linearized with *Sph*I (Klinger *et al.,* 1998). These 3′ half-plasmids are pSHIV-E-CAR, pSHIV-9466.33, pSHIV-9466su, pSHIV-E-R1, pSHIV-E-R2, pSHIV-A-170, and pSHIV-A-Q23. The *Sph*I-digested DNA samples, representing the 5′ and 3′ halves of proviral DNA, were mixed, ligated with T4 DNA ligase, and transfected into 293T cells by lipofection. Two days after transfection, 293T cells were cocultivated with human PBMC and cultured for 10–20 days. Supernatants from these cocultures were clarified by low-speed centrifugation and filtered through 0.2-μm filter and used for virus stocks. The titer of these stocks was determined by end-point dilution on PM-1 cells in microtiter plates.

Amounts of virus production from these stocks were determined by measuring SIV p27 core protein by ELISA (Coulter, Hialeah, FL).

Virus assay

After the 3-day stimulation period, cultures of human and rhesus macaque PBMC were inoculated with the viruses (SHIV-A-Q23, SHIV-A-170, SHIV-9466.33, pSHIV-9466su, SHIV-E-R2, or SHIV-E-CAR) at 0.001 $TCID_{50}$ per cell and maintained in complete RPMI 1640 medium supplemented with 50 u/ml IL-2. PM-1 and Mm221 cell lines were also exposed to the same amount of viruses. Culture supernatants were collected twice a week after infection and analyzed for virus production by measuring p27 SIV core protein by ELISA (Coulter).

Analysis of viral DNA by PCR amplification

PCR analysis was done to determine SHIV-9466.33 DNA in human and rhesus macaque PBMC. Primer pairs specific for the R/U5 region, S265 and S109, and the LTR/gag region of SIV_{mac239}, S265 and S267, were used to detect an early (295 bp) and a late (475 bp) reverse transcription product, respectively. The sequence of S109 is 5′ GATTTTCCTGCTTCGGTTTCCC 3′, and other primer sequences are cited in Zou and Luciw, 1996. Stimulated rhesus macaque PBMC and human PBMC, in the absence or presence of 10 μM AZT, were exposed to SHIV-9466.33 and SHIV-SF33 at 0.25 pg per cell and incubated at 37°C for 1.5 h. After absorption, cells were washed three times and maintained in culture medium supplemented with recombinant IL-2. Cell samples were collected at 0, 6, 12, and 24 h after infection and cell pellets were lysed in buffer containing 10 mM Tris (pH 8.3), 0.45% NP-40, 0.45% Tween 20, and 50 μg/ml proteinase K. To control for the amount of infected cell DNA substrate for PCR amplification, a primer pair (PC03 and PC04) specific for the human β-globin gene was included in all reactions (Saiki *et al.,* 1988). For detection of PCR amplification products, one primer from each pair of primers was end-labeled with [^{32}P]ATP by T4-polynucleotide kinase. Buffer for PCR amplification containing 1.5 mM $MgCl_2$, 50 mM KCl, 100 mM Tris pH 8.3 was mixed to 0.2 mM dNTP per nucleotide, 2 u *Taq* polymerase (Perkin–Elmer Cetus), and 20 pmol of the appropriate primers in a total volume of 50 μl. The PCR mixtures were subjected to 30 cycles of amplification by Thermal Cycler (Perkin–Elmer Cetus) at 94°C for 1.5 min and 72°C for 3 min, and extension at 72°C for 7 min. After the PCR amplification, DNA products were analyzed by electrophoresis on polyacrylamide gels and visualized by autoradiography of dried gels.

Fusion assay

Cell–cell fusion was assayed by using a fluorescence dye transfer system modified from Muñoz-Barroso *et al.* (1999). Briefly, 293T cells were transfected with 2 μg pND14-CAR, pND14–9466.33, pND14-R2, or pND14 (vector alone) by FuGENE-6 (Roche Molecular Biochemical, IN) in 100-mm culture dishes. Envelope-expressing 293T cells were replated at 6 h posttransfection onto coverslips in growth medium at 37°C. At 12 h posttransfection, the cells were labeled with 2 μM 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR: MW 554.04, ex/em 540/566 nm, Molecular Probes) for 30 min at 37°C. Subsequently, $CD4^+$ rhesus macaque T-lymphocyte Mm221 cells were labeled with 2 μM 5-chloromethylfluorescein diacetate (CMFDA: MW 484.86, ex/em 492/516, Molecular Probes) for 30 min at 37°C. The labeled cells were washed, resuspended in fresh medium, incubated for 30 min at 37°C, washed twice, and resuspended in growth medium. CMFDA-labeled macaque $CD4^+$ T-lymphocyte Mm221 cells and CMTMR-labeled *env*-expressing 293T cells were cocultured at a ratio of 10:1. After 8 h of cocultivation, the cells were examined using a Nikon Eclipse E800 epifluorescence microscope equipped with a CCD camera. CMTMR was visualized using an EF-4 G-2E/C TRITC filter cube (EX528–553; EM600–660). CMFDA was visualized using an EF-4 B-2E filter cube (EX450–490; EM520–560). Cells were also viewed under bright field to assess overlapping cells. For each experimental condition, an entire coverslip was examined using the 10× objective to assess the distribution of green and red fluorescence. Representative fields were then captured using the 20× or 40× objective. For each field, three images (bright field, CMTMR fluorescence, and CMFDA fluorescence) were captured directly into Photoshop. To confirm colocalization of red and green fluorescence within the same cell, the cocultures were also examined using a Zeiss LSM 510 laser scanning confocal microscope. Using a 40× oil objective, a stack of images with a step size of 0.9 μm and an optical section thickness of 0.9 μm was collected.

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