Virology **263,** 112–127 (1999)
Article ID viro.1999.9908, available online at http://www.idealibrary.com on IDEAL®

Fatal Immunopathogenesis by SIV/HIV-1 (SHIV) Containing a Variant Form of the HIV-1SF33 *env* Gene in Juvenile and Newborn Rhesus Macaques

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Received April 2, 1999; returned to author for final revision June 10, 1999; accepted July 20, 1999

SIV/HIV-1 (SHIV) chimeric clones, constructed by substituting portions of the pathogenic molecular clone SIVmac239 with counterpart portions from HIV-1 clones, provide a means to analyze functions of selected HIV-1 genes *in vivo* in nonhuman primates. Our studies focused on SHIVSF33, which contains the *vpu, tat, rev,* and *env* genes of the cytopathic, T-cell line tropic clone HIV-1SF33 (subtype-B); this clone has a premature stop codon in the *vpu* gene. In three juvenile macaques inoculated intravenously with SHIVSF33, low-level persistent infection was established; no disease was observed for a period of >2 years. However, at ~16 months p.i., one of four SHIVSF33-infected juvenile macaques exhibited an increase in virus load, depletion of $CD4^+$ T cells in peripheral blood and lymph nodes, and other symptoms of simian AIDS (SAIDS). Virus recovered from this animal in the symptomatic stage was designated SHIVSF33A (A, adapted); this virus displayed multiple amino acid sequence changes throughout the HIV-1 *env* gene compared with the input SHIVSF33 clone. Additionally, a mutation in all clones from SHIVSF33A restored the open reading frame for the *vpu* gene. *In vitro* evaluations in tissue-culture systems revealed that SHIVSF33A replicated to higher levels and exhibited greater cytopathicity than SHIVSF33. Furthermore cloned *env* genes for SHIVSF33A were more fusogenic in a cell-fusion assay compared with the *env* gene of the SHIVSF33. Intravenous inoculation of SHIVSF33A into juvenile and newborn macaques resulted in a rapid decline in $CD4^+$ T cells to very low levels and development of a fatal AIDS-like disease. A cell-free preparation of this pathogenic chimeric virus also established persistent infection when applied to oral mucosal membranes of juvenile macaques and produced a fatal AIDS-like disease. These studies on pathogenic SHIVSF33A establish the basis for further investigations on the role of the HIV-1 *env* gene in virus adaptation and in mechanism(s) of immunodeficiency in primates; moreover, the chimeric virus SHIVSF33A can play a role in elucidating mucosal membrane transmission and development of antiviral vaccines in newborns as well as juvenile and adult macaques.

INTRODUCTION

Infection of macaques with selected strains of simian immunodeficiency virus (SIV) is a valuable model for investigating many aspects of acquired immunodeficiency syndrome (AIDS) because of limitations of various animal models for human immunodeficiency virus type-1 (HIV-1) infection and disease (reviewed in Gardner and Luciw 1997). To address issues focused on the functions and immunological properties of the HIV-1 *env* gene in nonhuman primate models, chimeric viruses have been constructed between molecular clones of SIVmac and various strains of HIV-1 (Shibata *et al.,* 1991; reviewed in Overbaugh *et al.,* 1997). These chimeras, designated SHIV (Li *et al.,* 1992), contain an HIV-1 DNA fragment encoding *tat*, *rev*, *vpu,* and *env* cloned into the genome of the proviral form of SIVmac239, which is a clone that causes simian AIDS (SAIDS) in macaques (Kestler *et al.,* 1990). Pathogenic SHIV strains that produce $CD4^+$ T-cell depletion and an AIDS-like disease were produced by multiple serial passage of SHIV clones through macaques (Joag *et al.,* 1996; Reimann *et al.,* 1996). Accordingly, SHIV clones containing HIV-1 *env* genes exhibiting different phenotypes (e.g., cell tropism, coreceptor usage, cytopathicity) are being used to investigate *env*-mediated mechanisms of immunodeficiency and transmission through mucosal membranes and in vaccine studies (reviewed in Haga *et al.,* 1998; Ruprecht *et al.,* 1998; also see Kuiken 1997).

The present report describes the consequences of long-term infection of newborn and juvenile macaques with SHIVSF33, which contains the *env* gene of the T-cell tropic, cytopathic isolate HIV-1SF33. In a previous *in vivo* study, SHIVSF33 was shown to establish persistent infection in four juvenile macaques; after an initial peak of viremia in the acute phase (2–4 weeks p.i.), virus levels were low and all four animals remained healthy with no hematological or clinical abnormalities for an observation period of 1 year (Luciw *et al.,* 1995). However, one of these four juvenile macaques exhibited an increase in virus load at ~1.5 years after infection. This increase was accompanied by a dramatic decline in $CD4^+$ T cells and development of SAIDS. Virus recovered from this animal was designated SHIVSF33A (A, adapted). In a previous study, SHIVSF33A was shown to infect adult female rhesus

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0042-6822/99 $30.00

macaques by application of cell-free virus to vaginal mucosal membranes (Harouse *et al.,* 1998). In the present study, we examined the pathogenic properties of SHIV$_{SF33A}$ in both newborn and juvenile macaques and compared intravenous and oral mucosal membrane routes of inoculation. Additionally, sequence changes in the *env* gene of SHIV$_{SF33A}$ and *in vitro* replication properties of this virus also were analyzed. Understanding the effects of such sequence changes in HIV-1 genes in the chimeric virus will be important for defining molecular mechanisms that account for viral adaptation to AIDS pathogenesis in nonhuman primates.

RESULTS

Isolation of a pathogenic SHIV

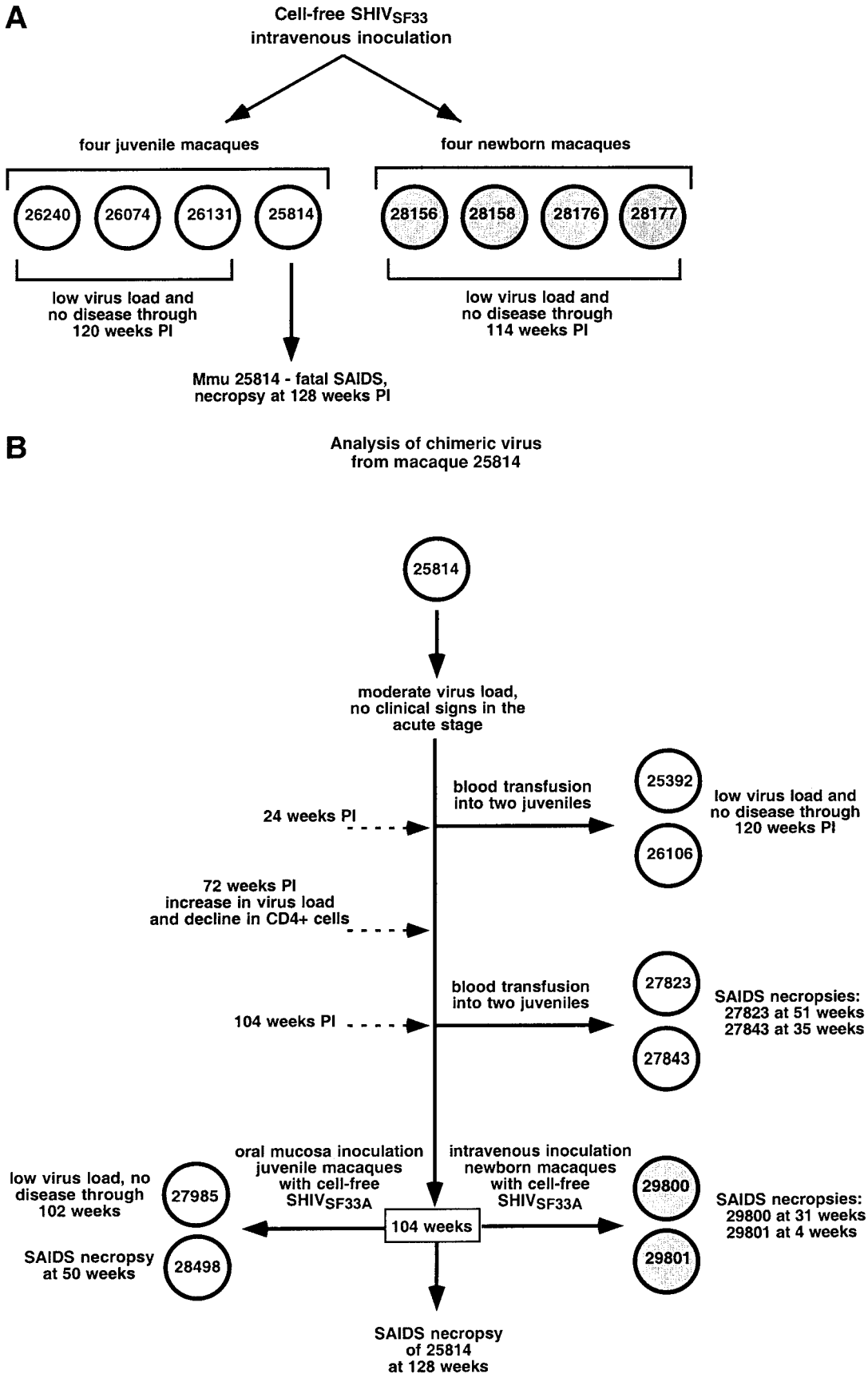
In a previous study, we inoculated four juvenile rhesus macaques (Mmu 25814, Mmu 26074, Mmu 26131, and Mmu 26240) with SHIV$_{SF33}$, a chimeric virus containing the *env* gp160 gene as well as the *vpu*, *tat,* and *rev* genes of the T-cell line tropic HIV-1$_{SF33}$ clone (Fig. 1A) (Luciw *et al.,* 1995). For an observation period of 1 year, virus load in peripheral blood and lymph nodes remained low in all four animals; no hematological abnormalities or other signs of disease were manifest (Luciw *et al.,* 1995). However, one of these macaques (Mmu 25814), exhibited an increase in virus load (Fig. 2A) with a concurrent decline in $CD4^+$ T cells in peripheral blood at 72 weeks after inoculation (Fig. 2B). For several weeks thereafter, virus load in Mmu 25814 was as high as in the two juvenile macaques (Mmu 26084 and Mmu 27098) infected with the pathogenic molecular clone SIVmac239nef+ (Fig. 2A). By 104 weeks, the number of $CD4^+$ T cells in Mmu 25814 declined to $<50/\mu$l (Fig. 2B), and this animal developed chronic diarrhea and lost ~20% of its body weight in the interval from 72 to 104 weeks. $CD8^+$ T-cell numbers in peripheral blood remained within the reference range (>1000 cells/μl) throughout the course of infection, whereas the CD4/CD8 T-cell ratio in both peripheral blood and lymph node biopsy samples dropped to <0.1 from 72 weeks onward (data not shown). In the macaques infected with the pathogenic molecular clone SIVmac239nef+, levels of $CD4^+$ T cells also declined to low levels in the late stage of infection (Fig. 2B).

Because of severe cachexia, Mmu 25814 was euthanized at 128 weeks. Major pathological findings at necropsy were disseminated lymphoid hyperplasia, lymphoid depletion in multiple lymph nodes, and interstitial pneumonia due to the opportunistic pathogen, *Pneumocystis carinii* (Table 1). Cellular distribution of viral RNA was determined in Mmu 25814 by a method combining *in situ* hybridization for viral RNA and immunohistochemistry for cell markers. In lymph node biopsies collected from Mmu 25814 at 92 weeks p.i., macrophages (HAM-56-positive cells) were the main cells expressing viral RNA; a much smaller proportion of $CD3^+$ T cells contained detectable viral RNA (Figs. 3A and 3B). The same pattern of localization of virus, largely in macrophages, was observed in a lymph node biopsy sample collected from Mmu 25814 at 118 weeks p.i. (data not shown). The other three juvenile macaques inoculated with SHIV$_{SF33}$ have remained healthy with normal hematological parameters and very low virus load for >2 years p.i. (data not shown).

Infection of juvenile macaques with pathogenic SHIV$_{SF33A}$

To determine whether the chimeric virus changed and acquired properties of a pathogenic virus during the course of infection in Mmu 25814, several inoculation studies were performed in rhesus macaques. Figure 1B illustrates the experimental plan, which included blood transfusion from Mmu 25814 to juvenile macaques and infection of juvenile and newborn macaques with cell-free virus isolated from Mmu 25814. Five milliliters of blood collected from Mmu 25814 at 24 and at 104 weeks was transfused into uninfected healthy juvenile macaques. The two juvenile animals (Mmu 25392 and Mmu 26106; Fig. 1B), receiving a blood transfusion from Mmu 25814 at 24 weeks p.i., showed low virus load, $CD4^+$ T cell levels >400 cells/μl, and no clinical signs throughout the 2-year period of observation (data not shown). In striking contrast, the two animals (Mmu 27823 and Mmu 27843; Fig. 1B) receiving a blood transfusion from Mmu 25814 at 104 weeks p.i. contained moderate levels of virus throughout infection (Fig. 4A) and exhibited a rapid decline in $CD4^+$ T cells in peripheral blood (Fig. 4B) and a dramatic reduction in CD4/CD8 ratios in lymph nodes (data not shown). Because $CD8^+$ T cells generally remained within the reference range, the CD4/CD8 ratio declined to <0.1 in the animals that showed the rapid decline in $CD4^+$ T cells (data not shown).

At necropsy at 35 weeks p.i., Mmu 27843 displayed widespread lymphoid hyperplasia and severe pulmonary infection with the opportunist, *Pneumocystis carinii* (Table 1). Necropsy analysis of the second animal, Mmu 27823, at 51 weeks p.i. revealed widespread lymphoid depletion and peritonitis with bacterial infections and abscesses in the abdominal cavity (Table 1). Cellular distribution of virus was examined by combined *in situ* hybridization and immunohistochemistry analysis of lymph node biopsies collected at 2, 8, 12, and 16 weeks p.i. from Mmu 27823 and Mmu 27843. Viral RNA was detected predominantly in macrophages (these authors, data not shown). Additionally, at necropsy, the main cell expressing viral RNA was the macrophage in lung (Figs. 3C and 3D), peripheral lymph nodes, gastrointestinal tract and gut-associated lymphoid tissue, and bone marrow (data not shown). Analysis of replication properties in cultures of human monocyte-derived macrophages revealed that SHIV$_{SF33A}$ replicated efficiently, whereas SHIV$_{SF33}$ produced very low levels of virus (data not shown.)


A
Cell-free SHIVSF33
intravenous inoculation
four juvenile macaques
four newborn macaques
26240
26074
26131
25814
28156
28158
28176
28177
low virus load and
no disease through
120 weeks PI
low virus load and
no disease through
114 weeks PI
Mmu 25814 - fatal SAIDS,
necropsy at 128 weeks PI
B
Analysis of chimeric virus
from macaque 25814
25814
moderate virus load,
no clinical signs in the
acute stage
24 weeks PI
blood transfusion
into two juveniles
25392
26106
low virus load and
no disease through
120 weeks PI
72 weeks PI
increase in virus load
and decline in CD4+ cells
104 weeks PI
blood transfusion
into two juveniles
27823
27843
SAIDS necropsies:
27823 at 51 weeks
27843 at 35 weeks
oral mucosa inoculation
juvenile macaques
with cell-free
SHIVSF33A
intravenous inoculation
newborn macaques
with cell-free
SHIVSF33A
low virus load, no
disease through
102 weeks
27985
SAIDS necropsy
at 50 weeks
28498
104 weeks
29800
29801
SAIDS necropsies:
29800 at 31 weeks
29801 at 4 weeks
SAIDS necropsy
of 25814
at 128 weeks

Cell-free virus, designated SHIV$_{SF33A}$, was obtained from PBMC of Mmu 25814 at 104 weeks (Fig. 1B). To investigate further the pathogenic potential of SHIV$_{SF33A}$ and to determine whether this virus could establish infection *via* transmission through mucosal membranes, the oral mucosal membranes of two juvenile macaques were exposed to a cell-free preparation of 26,000 $TCID_{50}$ of SHIV$_{SF33A}$ (Mmu 27985 and Mmu 28498; Fig. 1B). Both animals showed moderate levels of virus in the acute stage of infection; in the chronic stage, virus load was low for Mmu 28498 and very low for Mmu 27985 (Fig. 4A). Mmu 28498 exhibited a rapid and sustained decline of $CD4^+$ T cells in peripheral blood at 2 weeks onward, whereas $CD4^+$ T-cell levels remained >400 cells/μl in Mmu 27985 throughout the course of infection (Fig. 4B). Also, analysis of lymph node biopsies collected at 2 weeks and during the course of infection revealed a dramatic reduction in the CD4/CD8 ratio in Mmu 28498 but not in Mmu 27985 (data not shown). At ~10 months, Mmu 28498 developed chronic diarrhea and Bell's palsy; this animal was euthanized at 50 weeks p.i. Salient findings at necropsy of Mmu 28498 included widespread lymphoid depletion, thymic atropy, gastritis, and enteritis; multiple opportunistic pathogens were detected in this animal (Table 1).

SHIV$_{SF33A}$ infection and pathogenicity in newborn macaques

To evaluate the pathogenic potential of SHIV$_{SF33A}$ in newborn macaques, a cell-free preparation of this virus was inoculated into two newborn rhesus macaques by the intravenous route (Mmu 29800 and Mmu 29801; Fig. 1B). The infecting dose for each animal was 500 $TCID_{50}$ of virus. Virus load was high in PBMC at 1 week after infection and thereafter declined to relatively low levels in both animals (Fig. 5A). By 2 weeks, $CD4^+$ T-cell levels dropped to <50 cells/μl of peripheral blood in both newborn macaques (Fig. 5B). Mmu 29801 showed poor weight gain and was euthanized at 4 weeks p.i.; salient features at necropsy were lymphoid depletion, thymic atrophy, and severe meningioencephalitis and gastritis (Table 1). The brain pathology was suspected to be due to the viral opportunists rhesus cytomegalovirus (rhCMV) and simian vacuolating virus 40 (SV40). The second newborn, Mmu 29800, exhibited diarrhea and weight loss and was euthanized at 31 weeks p.i. (Table 1). Postmortem examination of Mmu 29800 revealed lymphoid hyperplasia, thymic atrophy, and severe eosinophilic bronchitis. This animal was also positive for the protozoal opportunist *Cryptosporidium*.

As a comparison for SHIV$_{SF33A}$ infection in newborn rhesus macaques, four additional newborn animals also were inoculated with a cell-free preparation of SHIV$_{SF33}$ by the intravenous route (Fig. 1A). These animals exhibited a relatively high virus load in the first 2 weeks p.i.; but virus load declined to low levels thereafter in all four animals (Fig. 5A). Additionally, these SHIV$_{SF33}$-infected newborn macaques maintained normal $CD4^+$ T-cell levels and did not develop any disease signs over an observation period of 2 years (Fig. 5B) (M. Marthas and P. Luciw, unpublished results). In summary, in newborn macaques SHIV$_{SF33A}$ was pathogenic, whereas SHIV$_{SF33}$ was relatively nonpathogenic.

Antiviral antibody responses in infected macaques

To establish whether macaques infected with SHIV$_{SF33}$ or SHIV$_{SF33A}$ produced antiviral immune responses, plasma antibodies were measured in an ELISA containing whole inactivated HIV-1 and HIV-2. SHIV$_{SF33A}$-infected Mmu 27985 did not demonstrate a rapid decline in $CD4^+$ T cells and contained readily detectable levels of antiviral antibodies, whereas the remaining SHIV$_{SF33A}$-infected animals exhibiting rapid $CD4^+$ T-cell decline after infection with this virus contained low or undetectable levels of antiviral antibodies (Table 2). It is possible that other serological methods, such as immunoblot or virus neutralization assay, may have detected antiviral antibodies in animals that were seronegative by the whole virus ELISA. For comparison, juvenile macaques infected with the pathogenic virus, SIVmac239, produced antiviral antibodies with titers, measured by ELISA, in a range similar to animals infected with SHIV$_{SF33}$ (Table 2).

Sequence changes in the HIV-1 *env* gene during SHIV infection

To determine whether changes occurred in HIV-1 sequences in SHIV$_{SF33}$ during the course of infection of Mmu 25814, the juvenile macaque displaying SAIDS (Fig. 1B; Table 1), the region encompassing the *tat*, *rev*, *vpu*, and *env* genes of SHIV$_{SF33A}$ was amplified by PCR, cloned, and sequenced; these sequences were submitted to GenBank. Figure 6 presents amino acid changes in six clones containing *env* gp120 sequences of SHIV$_{SF33A}$. Twenty-seven conservative and nonconservative amino acid changes were detected in the gp120 domain of all of these clones; most of these changes were in the variable regions. Three to four additional changes were unique to each clone. Interestingly, the V3 loop of the six clones gained a novel glycosylation site

FIG. 1. Scheme for inoculation of newborn and juvenile macaques with SHIV$_{SF33}$ and SHIV$_{SF33A}$. Rhesus macaques in this study were identified by a five-number code. Juvenile animals are represented by unfilled circles, and newborn animals are represented by shaded circles. (A) Four juvenile macaques (2–3 years of age) and four newborn macaques (2–3 days of age) were inoculated with SHIV$_{SF33}$. Juveniles and newborns received 1000 $TCID_{50}$ and 500 $TCID_{50}$, respectively, of a cell-free preparation of SHIV$_{SF33}$ by the intravenous route. (B) At 24 and 104 weeks after inoculation of Mmu 25814, whole blood was transfused from this animal into healthy juvenile macaques. At 104 weeks, virus designated SHIV$_{SF33A}$ was isolated from PBMC from Mmu 25814 and used to inoculate newborn macaques by the intravenous route and juvenile macaques by exposure of oral mucosa to cell-free virus.

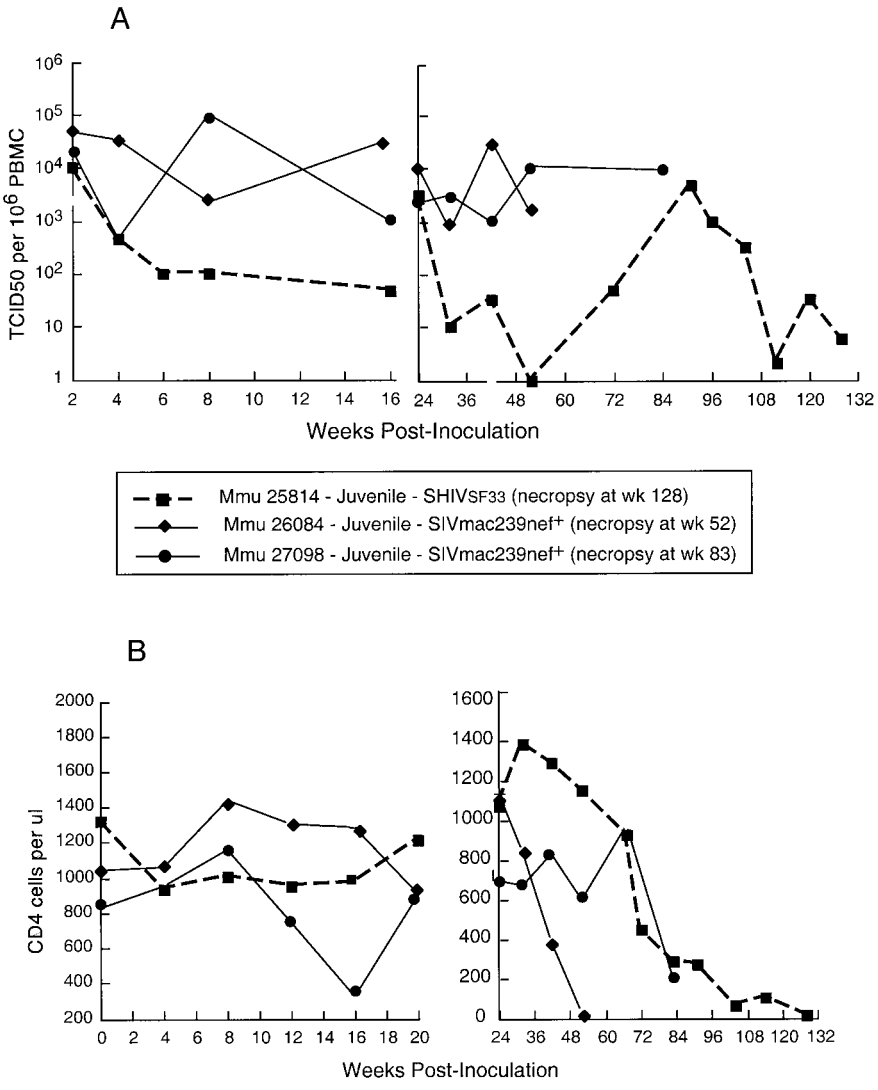


FIG. 2. Virus load and $CD4^+$ T-cell numbers in juvenile macaques inoculated with SHIVSF33. (A) Cell-associated virus load was monitored in PBMC collected from Mmu 25814 infected with SHIVSF33. Virus loads are also given for two juvenile macaques infected with the pathogenic molecular clone SIVmac239nef$^+$. (B) $CD4^+$ T-cell numbers in peripheral blood were determined by flow cytometry.

(Asn-Xaa-Ser/Thr) because the prototype sequence Asn-Asn-Arg was converted to Asn-Tyr-Thr. Also, all clones showed a change in the crown of the V3 loop from Gly-Pro-Gly-Lys to Gly-Pro-Gly-Arg. Other consistent changes in gp120 sequences were (i) the loss of a glycosylation site near the V1 region, (ii) a loss and gain of a glycosylation site in the V2 region in five of six clones, and (iii) a gain of a glycosylation site in the V5 region. Three of the six clones exhibited a loss of a glycosylation site in the V4 region. The transmembrane subunit of *env*, gp41, showed only three amino acid changes (Ala at 663 to Thr, Val at 618 to Ile, Ser at 678 to Gly) (submitted to GenBank). At 72 weeks p.i., the premature stop codon at position 2 in the *vpu* gene reverted to a codon for Gln (submitted to GenBank). A small number of amino acid changes were noted in *tat*, *rev*, *nef* and the LTR in some but not all SHIVSF33A clones examined (data not shown).

Amino acid sequence changes in the *env* gene also were determined for virus in Mmu 26240, which remained healthy with low virus load throughout the course of infection with SHIVSF33 (Fig. 1A). Analysis of five clones, containing the gp120 domain, from virus recovered from this animal at 2 years after inoculation, revealed three clones with no sequence changes and two clones with one (Val at position 256 to Met) or two changes (Glu at position 102 to Gly, Ser at position 210 to Pro) (submitted to GenBank). These changes in the two gp120 clones from Mmu 26240 did not affect potential glycosylation sites and did not match any changes in the six gp120 clones from SHIVSF33A (Fig. 6).

In vitro replication and cytopathology of SHIVSF33A

To explore potential differences in replication of the input virus SHIVSF33 and SHIVSF33A *in vitro*, cultures of macaque

TABLE 1

Clinical and Pathologic Findings in Rhesus Macaques Infected with SHIV$_{SF33}$ and SHIV$_{SF33A}$

Animal no. and age at inoculation	Route of inoculation[a]	Clinical signs and premortem laboratory data	Postmortem findings
Mmu 25814 39 mo.	Intravenous (cell-free SHIV$_{SF33}$)	CD4+ T-cell decline, lymphadenopathy, weight loss	Necropsy at 128 weeks p.i., lymphoid hyperplasia and depletion, lymphocytic interstitial pneumonia, enteritis, extensive bacterial colonization, *Pneumocystis carinii*
Mmu 27823 28 mo.	Transfusion (peripheral blood from Mmu 25814)	Rapid CD4+ T-cell decline, weight loss	Necropsy at 51 weeks p.i., lymphoid depletion in spleen and multiple lymph nodes, chronic active peritonitis, overwhelming, bacterial infections and abdominal abscesses
Mmu 27843 29 mo.	Transfusion (peripheral blood from Mmu 25814)	Rapid CD4+ T-cell decline, no weight gain, lymphadenopathy	Necropsy at 35 weeks p.i., lymphoid hyperplasia, severe pneumocystosis, *P. carinii*
Mmu 27985 32 mo.	Oral mucosa (cell-free SHIV$_{SF33A}$)	Normal CD4+ T-cell levels	Alive and healthy at 102 weeks p.i.
Mmu 28498 28 mo.	Oral mucosa (cell-free SHIV$_{SF33A}$)	Rapid CD4+ T-cell decline, low weight gain, diarrhea, intermittent high eosinophil levels, Bell's palsy	Necropsy at 50 weeks p.i., widespread lymphoid depletion, thymic atrophy, gastritis, enteritis, giant cell pneumonia, pneumocystosis, *Helicobacter, M. avium, P. carinii*
Mmu 29800 3 days	Intravenous (cell-free SHIV$_{SF33A}$	Rapid CD4 decline, diarrhea, weight loss, high eosinophil levels	Necropsy at 31 weeks p.i., lymphoid hyperplasis in spleen and multiple lymph nodes, thymic atrophy, *Cryptosporidium*
Mmu 29801 3 days	Intravenous (cell-free SHIV$_{SF33A}$)	Rapid CD4+ T-cell decline, dehydration, no weight gain, high eosinophil levels	Necropsy at 4 weeks p.i., lymphoid depletion, thymic atrophy, severe meningioencephalitis, severe gasritis

[a] Mmu 27823 and Mmu 27843 were inoculated by transfusing blood collected from SHIV$_{SF33}$-infected Mmu 25814 at 104 weeks p.i., (Fig. 1). All other macaques in this table were inoculated with a cell-free preparation of SHIV$_{SF33A}$ obtained from PBMC of Mmu 25814 at 104 weeks p.i., (Fig. 1).

PBMC and the CEMx174 cells were infected with cell-free stocks of these two viruses. SHIV$_{SF33}$(vpu+) was selected for this comparison because SHIV$_{SF33A}$ also encodes a full *vpu* translation frame. In both cell systems, SHIV$_{SF33A}$ exhibited more rapid replication kinetics and attained higher levels of virus in culture supernatants than SHIV$_{SF33}$vpu+ (Fig. 7). Additionally, observations by light microscopy revealed that cytopathic effects (i.e., vacuolization and syncytium formation) were detected earlier and to a greater extent in CEMx174 cells and PBMC cultures infected with SHIV$_{SF33A}$ than in cultures infected with SHIV$_{SF33}$vpu+ (data not shown).

To more directly examine viral cytopathology, two *env* gp120 genes cloned from SHIV$_{SF33A}$ (44.9 and 52-15, Fig. 6) were tested for fusogenic capacity in a cell-fusion assay. These two *env* clones displayed eight amino acid differences from each other, including differences in the V2, V3, and V4 loops. First, to compare levels of *env* glycoprotein expression, BHK-21 cells were transfected with mammalian cell expression plasmids containing the *env* gene of SHIV$_{SF33}$ (pND-33) or containing the *env* clones 44.9 (pND-33A1) and 52.15 (pND-33A2) (Fig. 6). At 48 h after transfection, lysates of transfected BHK-21 cells were prepared and analyzed by immunoblot for *env* glycoprotein expression. All three expression vectors produced similar amounts of *env* glycoprotein (Fig. 8A). Small differences in electrophoretic mobility were observed in the gp120 and gp160 forms between/among the *env* glycoproteins (Fig. 8A); these differences may be due to differences in glycosylation patterns. For the fusion assay, BHK-21 cells were transfected with each plasmid vector and cocultured with either CEMx174 or PM-1 cells at 48 h after transfection. Formation of multinucleate syncytia, detected by light microscopy at 9 h after initiation of the coculture, was four- to eightfold greater for the two SHIV$_{SF33A}$ *env* clones (pND-33A1 and pND-33A2) compared with the *env* gene of SHIV$_{SF33}$ (pND-33) (Fig. 8B). Thus *in vivo* passage of the chimeric viral clone resulted in altered *in vitro* phenotypes, which included augmented viral replication in macaque PBMC (Fig. 7A) and also enhanced fusogenicity of the *env* gene (Fig. 8B).

DISCUSSION

SHIV pathogenesis in juvenile and newborn macaques

This paper reports the derivation and characterization of a pathogenic chimeric virus, SHIV$_{SF33}$, which contains the *env* region of the T-cell line tropic HIV-1$_{SF33}$ virus on

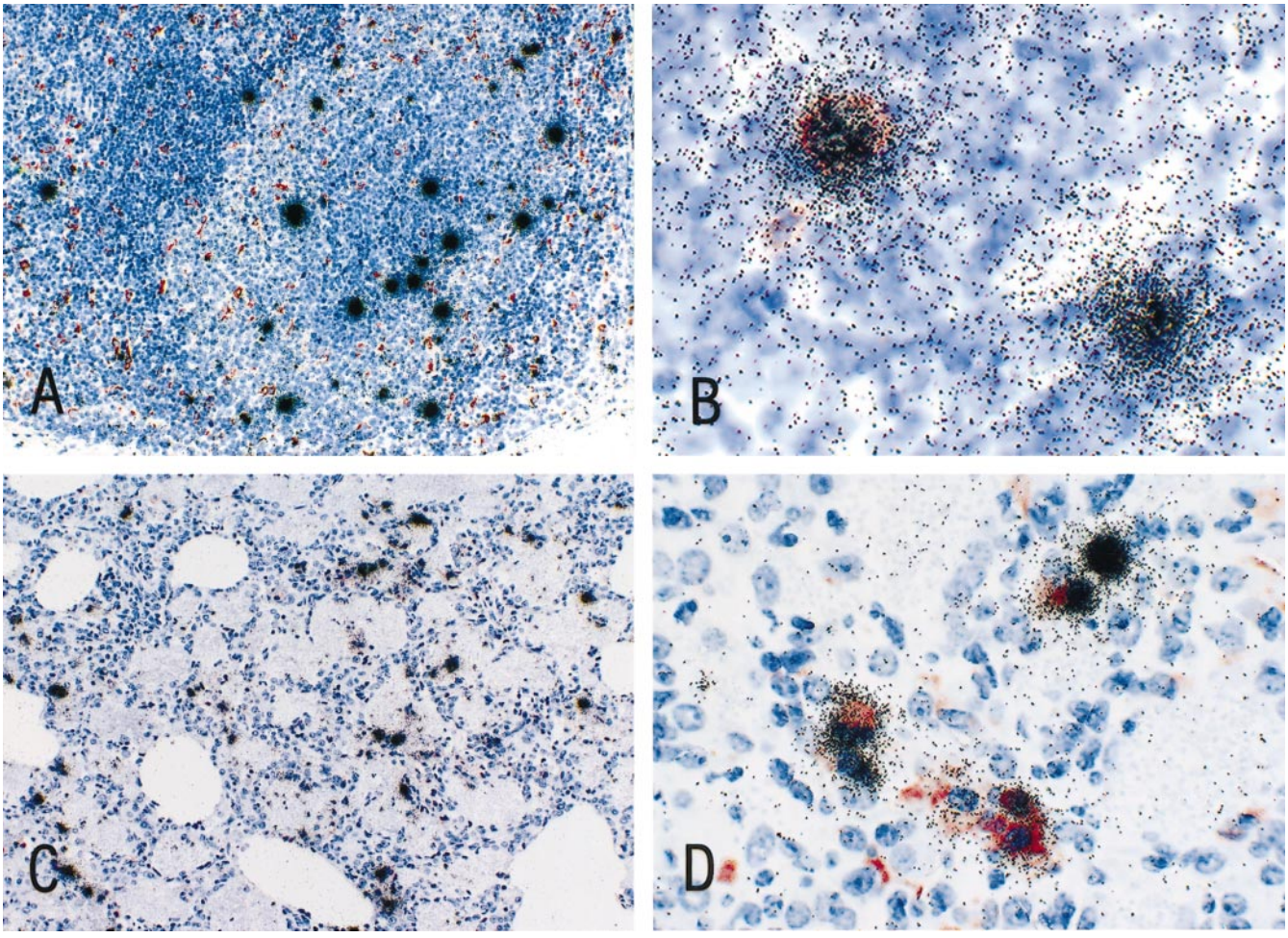


FIG. 3. Cellular localization of viral nucleic acid in SHIV infected macaques. (A) A peripheral lymph node, collected from Mmu 25814 at 92 weeks after infection with SHIV$_{SF33}$, shows the localization and distribution of viral nucleic acid (black silver grains representing *in situ* hybridization signal for SHIV nucleic acid) at low magnification (200X). (B) At higher magnification (×500), this same lymph node shows the colocalization of viral RNA by *in situ* hybridization in macrophages immunohistochemically labeled with the macrophage cytoplasmic marker HAM-56 (red-orange AEC chromogen). (C) Lung was collected from Mmu 27843 at necropsy at 35 weeks after infection of this animal by blood transfusion from Mmu 25814 (Fig. 1B); this section shows distribution of viral nucleic acid (silver grains at ×200 magnification). (D) The same lung tissue, viewed at higher magnification (×500), reveals cells doubly labeled with HAM-56 for macrophages (AEC chromogen) that contain viral nucleic acid (black silver grains).

the background of the pathogenic SIVmac239 clone. This chimera utilizes the CXCR4 coreceptor for entry into cells (Chakerian *et al.,* 1997). One of four juvenile rhesus macaques inoculated with SHIV$_{SF33}$ developed an AIDS-like disease commencing at 72 weeks p.i. Virus recovered from this animal, designated SHIV$_{SF33A}$, produced fatal immunodeficiency in juvenile and newborn macaques after inoculation by intravenous or mucosal membrane routes. In contrast, the chimeric clone SHIV$_{HXB2}$ (containing the *env* region of T-cell line tropic HIV-1 $_{HXB2}$) required multiple serial passage in nemestrina macaques to produce pathogenic virus (SHIV$_{KU}$-1) (Joag *et al.,* 1996). Also, multiple serial passage of SHIV89.6 (containing the *env* gene of dualtropic HIV-189.6) in rhesus macaques was important for generating a pathogenic virus (SHIV89.6$_P$) (Reimann *et al.,* 1996). It is possible that host differences, such as immunogenetic factors, may have accounted for the finding that the derivation of pathogenic SHIV$_{SF33A}$ did not require multiple passages in macaques. Accordingly, a likely scenario is that the animal Mmu 25814 allowed sufficient levels of replication of the SHIV$_{SF33}$ inoculum during the chronic phase of infection to generate a viral variant(s) that produced immunodeficiency. Age is a host factor that influences disease progression in HIV-1-infected patients (Mueller and Pizzo 1997) and SIV-infected macaques (Marthas *et al.,* 1995). Similarly, we observed that SHIV$_{SF33A}$ produced more rapid disease progression in newborn macaques than juvenile animals.

Host genetic differences also may account for the fact that not all animals infected with the same virus exhibited the same pattern of virus load and disease progression (Bontrop *et al.,* 1996; Sauermann *et al.,* 1997). Consistent with the idea that host factors influence infection

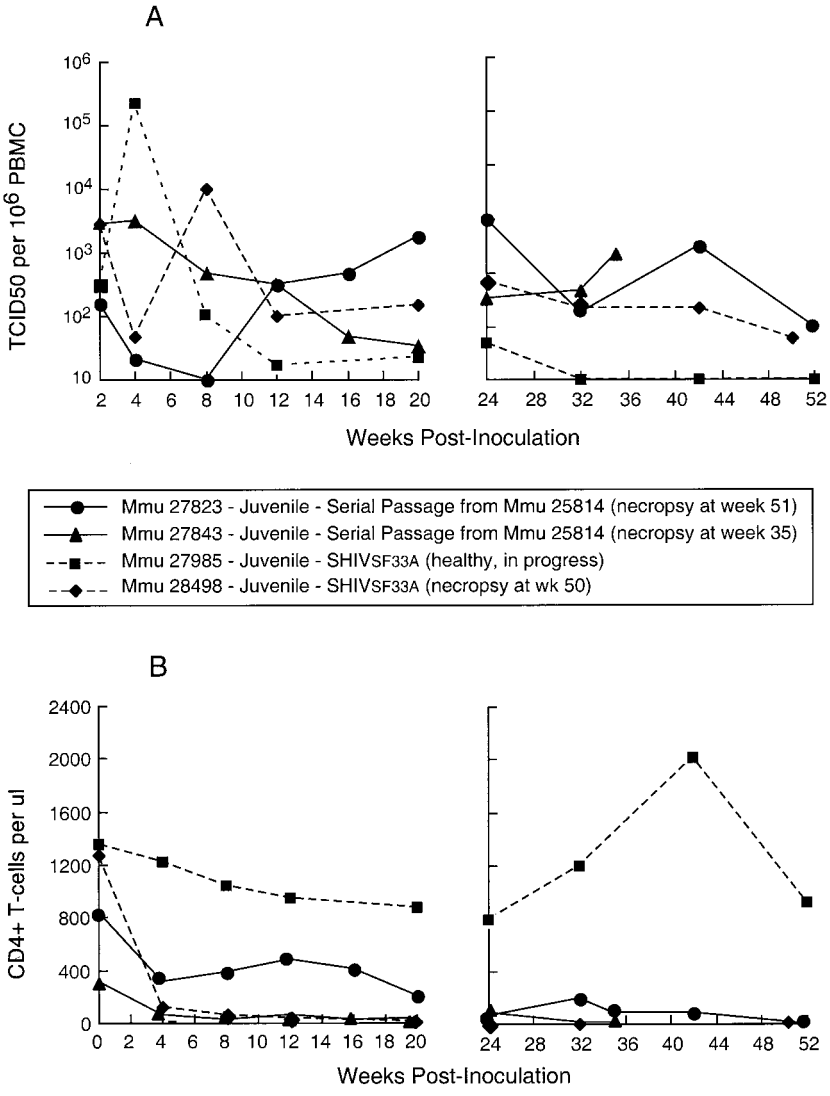


FIG. 4. Virus load and $CD4^+$ T-cell numbers in juvenile macaques inoculated with pathogenic SHIV. (A) Mmu 27823 and Mmu 27843 received a blood transfusion (5 ml) from Mmu 25814 at 104 weeks p.i. Mmu 27985 and Mmu 28498 were inoculated by exposure of oral mucosa to cell-free SHIVSF33A. For all of these animals, cell-associated virus load was monitored in PBMC. (B) $CD4^+$ T-cell numbers in peripheral blood were determined by flow cytometry.

with chimeric virus is the observation that macaques infected with SHIV constructs containing the *env* gene of HIV-1DH12 displayed different patterns of $CD4^+$ T-cell loss (Shibata *et al.,* 1997). In two juvenile macaques infected with SHIVSF33A by the oral mucosal route in our study, one animal (Mm 28498) showed a rapid $CD4^+$ T-cell decline, moderate virus loads, and simian AIDS, whereas the second animal (Mm 27985) survived for >2 years with normal levels of $CD4^+$ T cells, low virus loads, and no clinical signs of immunodeficiency disease. In a previous study, some female macaques infected with SHIVSF33A *via* vaginal mucosal membranes showed a rapid decline in $CD4^+$ T cells, whereas others retained normal levels of this cell subset (Harouse *et al.,* 1998). Alternatively, it is possible that inoculation across mucosal membranes selects for viral variants that differ in pathogenic potential (Lu *et al.,* 1996; Trivedi *et al.,* 1996).

A major feature of infection of macaques with pathogenic SHIV is rapid and severe depletion of $CD4^+$ T cells; this depletion occurs in most animals infected with these chimeric viruses (Joag *et al.,* 1996; Reimann *et al.,* 1996; Steger *et al.,* 1998). In contrast, infection with SIVmac239 or other strains of SIV generally produces little or no effect on total number of $CD4^+$ T cells throughout the course of infection, except in terminal stages of simian AIDS when this subset of T cells declines (Reimann *et al.,* 1994). The basis for the rapid loss of $CD4^+$ T cells in pathogenic SHIV infection is not known. A potential explanation is that pathogenic SHIV isolates are more cytopathic for $CD4^+$ T cells *in vivo* than either SIVmac239 or nonpathogenic SHIV isolates. Alternatively, pathogenic SHIV isolates might more profoundly effect precursors of $CD4^+$ T cells, thereby preventing replenishment of cells in this subset (Ho *et al.,* 1995; Wei

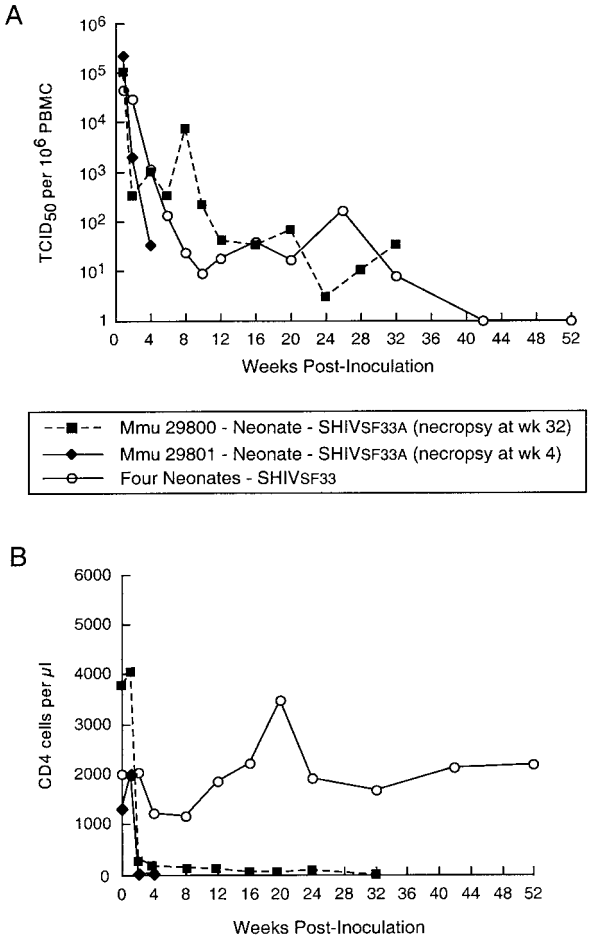


FIG. 5. Virus load and $CD4^+$ T-cell numbers in $SHIV_{SF33}$ and $SHIV_{SF33A}$-infected newborn macaques. (A) Cell-associated virus load was monitored in PBMC collected from two newborns inoculated with $SHIV_{SF33A}$ and four newborn macaques inoculated with $SHIV_{SF33}$ (mean virus load for four animals). (B) $CD4^+$ T-cell numbers in peripheral blood were determined by flow cytometry for two newborns inoculated with $SHIV_{SF33A}$ and four newborns infected with $SHIV_{SF33}$ (mean $CD4^+$ T-cell counts for four animals).

et al., 1995; Rosenzweig *et al.,* 1998). The enhanced ability of $SHIV_{SF33A}$ to infect and replicate in cultures of rhesus lymphoid cells and to produce cytopathic effects in such cultures may provide an explanation for the pathogenic nature of this virus compared with the input virus $SHIV_{SF33}$. Although direct killing of $CD4^+$ T cells *in vivo* by pathogenic SHIV may account for the rapid and sustained depletion of this lymphocyte subset, it is also possible that an indirect mechanism(s), such as disruption of lymphocyte homeostatic mechanisms in the host, may contribute to $CD4^+$ T-cell depletion (Pantaleo and Fauci, 1996).

Viral sequence changes during *in vivo* passage of SHIV

A key issue to understanding the determinant(s) of SHIV adaptation to viral persistence, $CD4^+$ T-cell depletion, and disease is the sequence change(s) in the chimeric virus during *in vivo* passage. Such a change(s) might confer increased infectivity of the SHIV for macaque target cells (e.g., $CD4^+$ T cells, monocytes/macrophages, and/or dendritic cells) or enable the virus to escape the host immune response. Both of these mechanisms for SHIV adaptation to pathogenesis are not mutually exclusive.

Comparisons of $SHIV_{SF33}$ and $SHIV_{SF33A}$ revealed amino acid changes in $HIV\text{-}1_{SF33}$ *vpu*, *tat*, *rev*, and both subunits of *env*. Other investigators also have reported a small number of substitutions in *vpu*, *tat*, and *rev* for the pathogenic isolates $SHIV_{KU}$-1 and $SHIV89.6_P$ (Karlsson *et al.,* 1997; Stephens *et al.,* 1997). Restoration of the *vpu* translation frame in $SHIV_{SF33A}$ was probably important for viral persistence. However, this reversion in *vpu* is not the sole determinant for pathogenesis; a previous study of SHIV-4 (constructed from $HIV\text{-}1_{HXB}2$) revealed that *vpu* played a role in viral persistence but was not sufficient to cause simian AIDS (Li *et al.,* 1995). The changes in *tat* and *rev* generally involved synonymous substitutions;

TABLE 2

Levels of Antiviral Antibodies in Juvenile and Newborn Macaques Infected with $SHIV_{SF33}$ or $SHIV_{SF33A}$

Animal	Inoculum	Weeks postinfection[a]	Titer[b]
Mmu 25814 (juvenile)	$SHIV_{SF33}$	52	1/102,400
		128 (necropsy)	1/12,800
Mmu 28156 (newborn)	$SHIV_{SF33}$	52	1/800
Mmu 28158 (newborn)	$SHIV_{SF33}$	52	1/12,800
Mmu 28176 (newborn)	$SHIV_{SF33}$	52	1/3,200
Mmu 28177 (newborn)	$SHIV_{SF33}$	52	1/3,200
Mmu 27823 (juvenile)	Transfusion from Mmu 25814	24	1/200
		51 (necropsy)	1/200
Mmu 27843 (juvenile)	Transfusion from Mmu 25814	24	<1/100
		35 (necropsy)	<1/100
Mmu 29800 (newborn)	$SHIV_{SF33A}$	31 (necropsy)	1/800
Mmu 29801 (newborn)	$SHIV_{SF33A}$	2	<1/100
Mmu 27985 (juvenile)	$SHIV_{SF33A}$	24	1/3,200
		52	1/3,200
Mmu 28498 (juvenile)	$SHIV_{SF33A}$	24	<1/100
		50 (necropsy)	<1/100
Mmu 26084 (juvenile)	SIVmac239	24	1/3,200
		51 (necropsy)	1/6,400
Mmu 27098 (juvenile)	SIVmac239	24	1/1,024,000
		83 (necropsy)	1/819,200

[a] Weeks after inoculation with cell-free virus or after blood transfusion.

[b] Levels of antiviral antibodies in plasma samples were measured in a whole virus ELISA containing a mixture of HIV-1 and HIV-2 antigens (Genetics Systems, Seattle, WA). Titers shown as <1/100 are below the detection level of the ELISA.

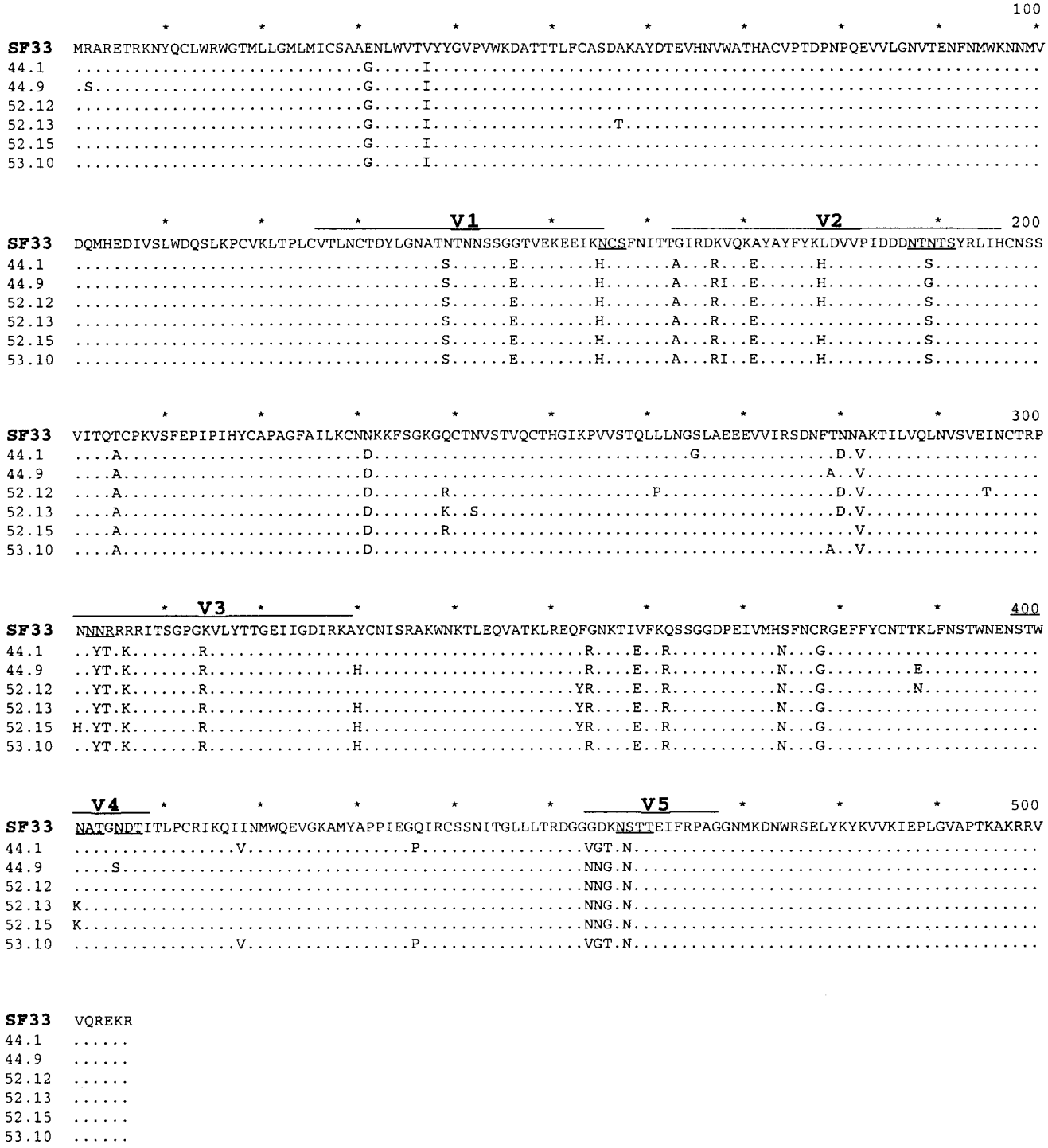


FIG. 6. Sequence changes in the SHIV$_{SF33A}$ envelope glycoprotein. SHIV$_{SF33A}$ is the virus obtained from Mmu 25814 at 104 weeks after inoculation (Fig. 1B). The top line shows the sequence of the gp120 *env* subunit of HIV-1$_{SF33}$ in the one letter amino acid code. This figure shows differences in sequences for six clones representing the gp120 domain of SHIV$_{SF33A}$. Variable regions V1 to V5 are overlined. Potential glycosylation sites (N-X-S/T) are underlined. DNA sequences are deposited in GenBank.

additionally, these changes were different for the three pathogenic SHIV isolates.

For SHIV$_{SF33A}$, most of the amino acid sequence substitutions were in the *env* gene, and the majority of these were in the variable regions of the gp120 subunit. SHIV$_{KU}$-1 and SHIV89.6$_P$ both exhibited 10 changes in gp120 when compared with each of their predecessor clones SHIV-4 and SHIV89.6, respectively; however, these changes were different for both of these viruses and did not match the 24 amino acid changes acquired

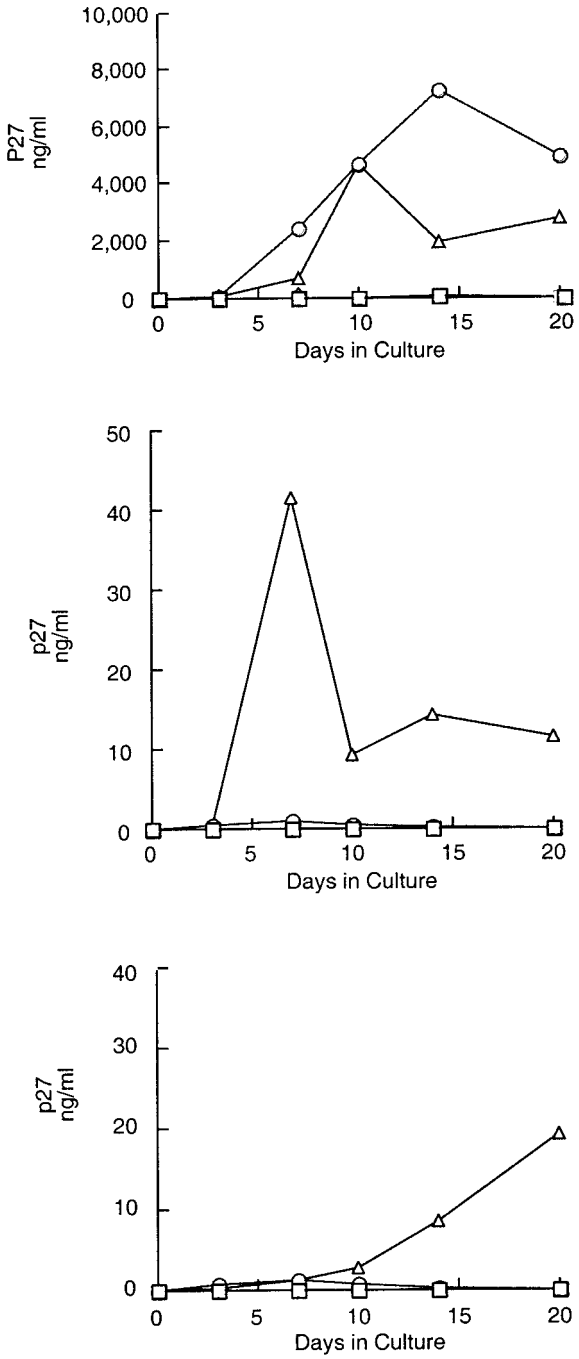


FIG. 7. *In vitro* replication of chimeric viruses. Comparisons were made in the replication of SHIVSF33vpu+ and SHIVSF33A. Cultures of CEMx174 cells (A) and stimulated PBMC from two uninfected macaques (Mmu 23587 and Mmu 23020; B and C, respectively) were infected with 1000 $TCID_{50}$ of each virus per 10^6 cells. Uninfected cultures, designated "mock," were included for each cell type. Samples of culture media were collected and assayed for SIV p27gag by ELISA at 3- to 4-day intervals. □, Mock infection; ○, SHIV-33vpu+; △, SHIV-33A.

by SHIVSF33A. A recent structural model of the gp120 subunit identified several amino acid residues, highly conserved among numerous HIV-1 subtype-B isolates, which are located in the site for attachment to the CD4 antigen and in the site for interaction with the coreceptor on cell membranes (Kwong *et al.,* 1998; Rizzuto *et al.,* 1998). None of the substitutions in the gp120 subunit of these three pathogenic SHIV isolates involved substitutions in these residues. The structural model also predicts that the V1 and V2 loops play a role in attachment to the coreceptor. Additionally, genetic studies of viral mutants, analyzed in tissue culture systems, also indicate that the V3 loop influences coreceptor usage (Cocchi *et al.,* 1996; Wu *et al.,* 1996). Thus the changes in the V1, V2, and V3 loops, which occurred during *in vivo* passage of each chimeric virus, could influence coreceptor usage in a qualitative and/or quantitative fashion and could consequently affect cell tropism (Doms and Moore 1997; Platt *et al.,* 1998).

Although all three pathogenic SHIV isolates (SHIVSF33A, SHIVKU-1, and SHIV89.6P) contained substitutions in the V3 epitope, only SHIVSF33A acquired a (potential) glycosylation site in this loop. Previous investigators demonstrated that presence of a glycosylation site in the HIV-1 V3 loop enhanced resistance of virus to neutralizing antibody (Back *et al.,* 1994; Schonning *et al.,* 1996) and altered the efficiency of coreceptor usage (Nakayama *et al.,* 1998). Further studies are required to determine whether the new (potential) glycosylation site in V3 of SHIVSF33A affects virus neutralization by antibody and/or a *env*-mediated function in viral replication. Nonetheless other changes in V3, as well as other regions of *env*, could be a consequence of immune selection by the host (McMichael *et al.,* 1997).

All three pathogenic chimeras (SHIVSF33A, SHIVKU-1, and SHIV89.6P) acquired three to four amino acid changes in the gp41 subunit that were unique to each virus. In addition, SHIV89.6 exhibited a deletion at the carboxyl-terminus of gp41 that removed 42 amino acid residues (Karlsson *et al.,* 1997). This deletion joined the translation frame for HIV-1 gp41 with the portion of the carboxyl-terminus of SIVmac239 gp41 that overlaps the *nef* gene (57 amino acid residues) to produce a hybrid transmembrane glycoprotein. SHIVSF33A and SHIVKU-1 did not show such a drastic alteration in the *env* transmembrane glycoprotein (Fig. 6; Stephens *et al.,* 1997); this finding indicates that the deletion event producing the hybrid gp41 subunit is unique to SHIV89.6P and may not be important for viral adaptation and pathogenesis (Karlsson *et al.,* 1998).

Investigation of *env* determinants of SHIV pathogenesis

Pathogenic SHIV isolates are being used to analyze the roles of various HIV-1 *env* genes in the virus/host relationship and as challenge viruses in vaccine studies in nonhuman primate models for lentivirus infection and simian AIDS (reviewed in Overbaugh *et al.,* 1996; Warren and Levinson 1997). SHIVSF33A, similar to other pathogenic chimeric viruses, causes a rapid and sustained

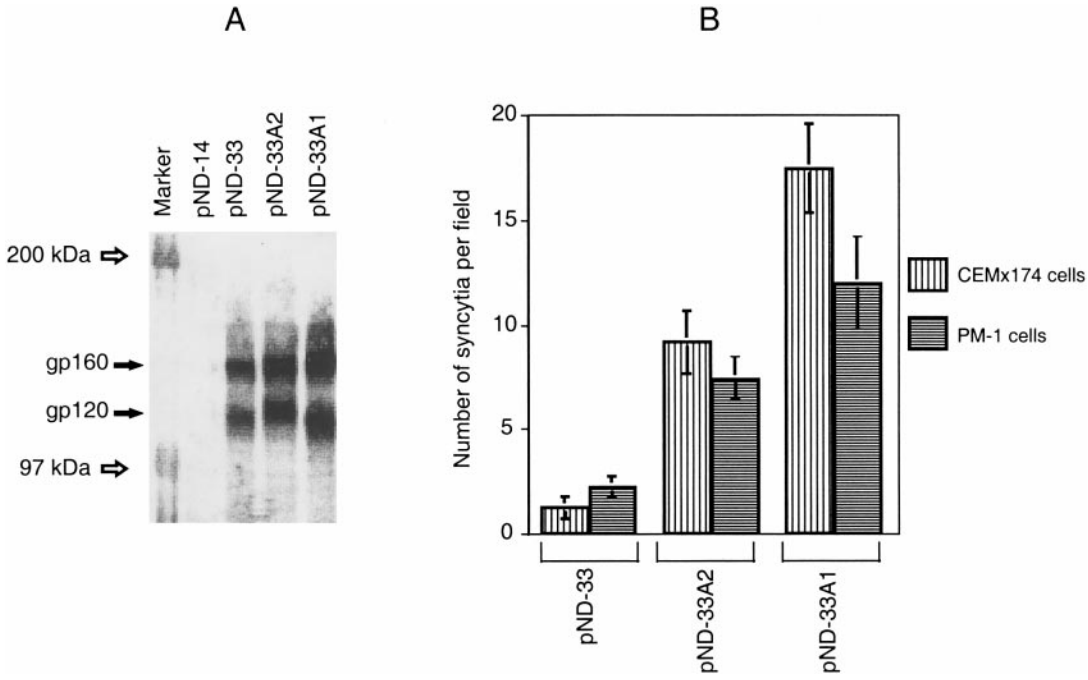


FIG. 8. Cell fusion assay for SHIV$_{SF33}$ and SHIV$_{SF33A}$ *env*. The mammalian cell expression vector pND-33 contains the *env* gene of SHIV$_{SF33}$, and the vectors pND-33A1 and pND-33A2 contain the *env* clones 44.9 and 52.15, respectively (Fig. 6). Cultures of BHK-21 cells, in 35-mm plates for immunoblot analysis or in 24-well microtiter plates for fusion assay, were transfected with each of these three expression plasmids or pND-14 (empty vector control). (A) Levels of *env* glycoproteins (gp160 precursor and gp120 surface domain) in BHK-21 cell lysates, prepared at 48 h after transfection, were assessed by electrophoresis on 8% polyacrylamide gels followed by immunoblot analysis with anti-HIV-1 *env* gp120 polyclonal antibody. (B) At 48 h after transfection, CEMx174 or PM-1 cells were added to BHK-21 cell cultures (in 24-well microtiter plates) previously transfected with each plasmid vector (quadruplicate wells for each plasmid vector). Multinucleate syncytia, containing 4 or more nuclei, were detected by light microscopy (×100 magnification) at 9 h after the start of the coculture. No syncytia were detected in transfections with the control vector pND-14 (data not shown). The values given for each plasmid vector represent the average obtained from counting syncytia in a total of six fields per well (four wells per plasmid vector). Error bars indicate standard deviation.

depletion in $CD4^+$ T cells, and this depletion occurs in animals infected with cell-free virus by the intravenous route as well as by vaginal (Harouse *et al.,* 1998) or oral mucosa (Joag *et al.,* 1997; Lu *et al.,* 1998). Thus this pair of chimeric viruses, SHIV$_{SF33}$ and SHIV$_{SF33A}$, can be used to examine the role of the HIV-1 *env* gene in viral pathogenesis and transmission through mucosal membranes. Comparisons of sequences of three pathogenic chimeras (i.e., SHIV$_{SF33A}$, SHIV89.6$_P$, and SHIV$_{KU}$-1) have not revealed a simple picture of the amino acid changes in the *env* glycoprotein that correlate with conversion of nonpathogenic to pathogenic virus. Further studies with point mutants and intragenic recombinants in the gp120 subunit of *env* will be necessary to determine which change(s) is (are) important for adaptation and disease in the macaque host.

A measurable phenotype(s) based on an *in vitro* assay (i.e., in tissue culture) will be important for elucidating molecular mechanisms of SHIV immunodeficiency. Accordingly, further studies are required to determine whether the augmented replication and cytopathicity of SHIV$_{SF33A}$ is important for the rapid $CD4^+$ T-cell decline *in vivo*. The pathogenic SHIV89.6$_P$ contains an *env* gene that also displays higher fusion capacity and increased coreceptor binding capacity than the nonpathogenic SHIV89.6 (Karlsson *et al.,* 1998). Both SHIV$_{SF33}$ and SHIV$_{SF33A}$ utilize the CXCR4 coreceptor (Harouse *et al.,* 1998); it is possible that these two viruses may differ in the usage of other (novel) coreceptors in macaques (Farzan *et al.,* 1997; Hoffman *et al.,* 1998). Interestingly, pathogenic SHIV$_{KU}$-1 differed from its nonpathogenic predecessor by exhibiting higher levels of replication in macaque macrophages (Stephens *et al.,* 1997). Potential differences in cell tropism between SHIV$_{SF33}$ and SHIV$_{SF33A}$ remain to be determined. It is possible that sequence changes in *env* also may enable virus to escape host immune responses (McMichael and Philips 1997). Evolution of HIV-1 in humans and SIV in susceptible macaques involves changes in viral phenotypes (coreceptor usage, cytopathicity) and antigenic properties, and these changes appear to correlate with disease progression (Connor *et al.,* 1994; Wolinsky *et al.,* 1996; Rudensey *et al.,* 1998). The results in this study, on the nonpathogenic and pathogenic pair SHIV$_{SF33}$ and SHIV$_{SF33A}$, respectively, taken together with reports on such pairs of other SHIV isolates (Joag *et al.,* 1996; Reimann *et al.,* 1996; Karlsson *et al.,* 1998), set the stage for analyzing functions of the HIV-1 *env* glycoprotein and its domains in viral pathogenesis and for studying antiviral immune responses in nonhuman primate models for AIDS.

MATERIALS AND METHODS

Cells and viruses

Peripheral blood mononuclear cells (PBMC) were obtained from healthy rhesus macaques free of simian type-D retroviruses (SRV), SIV, and simian T-lymphotropic virus (STLV). These cells, purified from whole blood by Ficoll–Hypaque centrifugation, were stimulated with staphylcoccal enterotoxin A (SEA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS) and 10% interleukin-2 (IL-2) (Chiron Corp., Emeryville, CA) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). CEMx174 cells, a human hybrid T–B-cell line (provided by Dr. J. Hoxie, University of Pennsylvania, Philadelphia, PA), were maintained in RPMI 1640 medium supplemented with 5% calf serum plus 5% fetal calf serum and antibiotics. PM-1 cells (obtained from Dr. R. Gallo, University of Maryland, Baltimore, MD) are a human T-cell line permissive for primary as well as T-cell line adapted strains of HIV-1 (Lusso *et al.,* 1995). MM221 cells (provided by Dr. R. Desrosiers, New England Regional Primate Research Center, Southboro, MA) are an IL-2-dependent rhesus macaque T-cell line permissive for SIV and SHIV (Alexander *et al.,* 1997). BHK-21 (baby hamster kidney cells) were obtained from the American Type Culture Collection (Rockville, MD).

The stock of cell-free SHIV$_{SF33}$ was previously described (Luciw *et al.,* 1995). SHIV$_{SF33}$vpu+ was produced by changing the premature stop codon at position 2 in *vpu* to a glutamine codon by site-directed mutagenesis (C. Cheng Mayer). Both SHIV$_{SF33}$vpu+ and SHIV$_{SF33A}$ were propagated in a large culture of CEMx174 cells. Culture supernatants were collected at 5–6 days p.i. (when ~25% of cells displayed cytopathic effects), passed through a 0.22-μm filter, and frozen in 1-ml aliquots. The 50% tissue culture infectious dose ($TCID_{50}$) of these viral stocks in CEMx174 cells was determined by end-point dilution in microtiter plates as described previously (Marthas *et al.,* 1993).

Rhesus macaques and viral inoculations

All animals were colony-bred juvenile rhesus macaques (*Macaca mulatta*) free of SRV, SIV, and STLV; these animals are housed at the California Regional Primate Research Center (CRPRC) at Davis, CA, in accordance with American Association for Accreditation of Laboratory Animal Care Standards. Neonatal macaques, born to dams seronegative for SRV, SIV, and STLV, were removed from their mothers and reared in a primate nursery. Before inoculation, 20 ml of blood from juvenile macaques and 1 ml from newborn macaques (2–3 days of age) was collected by venipuncture for complete blood count (CBC) including platelet count, CD4/CD8 T lymphocyte phenotyping by flow cytometry, and for preinfection plasma and PBMC samples. From juvenile macaques, peripheral lymph nodes were obtained by excisional biopsy, and portions of lymph nodes were fixed in formaldehyde and OCT. The scheme for inoculating juvenile and neonatal macaques is shown in Fig. 1. Juvenile macaques received a 5-ml blood transfusion from Mmu 25814 at 24 and 104 weeks p.i. For transmission across mucosal membranes, oral mucosa of juvenile macaques were exposed to 1 ml of cell-free SHIV$_{SF33A}$ containing 26,000 $TCID_{50}$. Neonatal macaques were inoculated with 500 $TCID_{50}$ of 0.5 ml of cell-free SHIV$_{SF33A}$ by the intravenous route. Animals were observed daily and weighed regularly by the CRPRC veterinary staff. Complete physical examinations were performed to monitor for weight loss, lymphadenopathy, and/or splenomegaly, opportunistic infections as well as any other clinical signs of disease. Animals meeting three of the following criteria were euthanized: (i) weight loss >10% in 2 weeks or 30% in 3 months, (ii) chronic diarrhea unresponsive to treatment, (iii) infections unresponsive to antibiotics, (iv) inability to maintain body heat or fluids without supplementation, (v) persistent, marked hematological abnormalities including lymphopenia, anemia, thrombocytopenia, or neutropenia, and (vi) persistent, marked splenomegaly or hepatomegaly. A complete necropsy was performed on each euthanized animal; this included gross and microscopic examination of tissues.

Measures of viral load

Levels of viral antigen in plasma were measured with an ELISA kit for SIV $p27^{gag}$ antigen (Coulter Immunology, Hialeah, FL). For measuring plasma viremia, serial 10-fold plasma dilutions were made in tissue culture medium and dispensed into 24-well microtiter plates containing 2.5×10^5 CEMx174 cells (Marthas *et al.,* 1993). Cultures in these plates are passaged every 3–4 days over a 4-week period. Four replicates were made for each dilution. Growth of virus is scored by microscopic observation of cytopathology and by measuring viral antigen in tissue culture supernatant with the SIV $p27^{gag}$ ELISA kit. For measuring cell-associated viral loads, 10^6 PBMC or lymph node cells (and serial 10-fold dilutions of these cells) from each infected macaque were cocultured with 2.5×10^5 CEMx174 cells per well with four wells per dilution (Marthas *et al.,* 1993). These cocultures were monitored by light microscopy for cytopathology, and samples of media were assayed for SIV $p27^{gag}$ antigen by the ELISA kit to monitor virus production. Titers were calculated by the method of Reed and Meunch to determine the number of infected PBMC per 10^6 total PBMC. To measure virus load in lymph nodes, peripheral lymph nodes were obtained by transcutaneous biopsy and aseptically teased into single-cell suspensions; cell numbers were determined by counting in a hemocytometer.

Hematological evaluation and CD4/CD8 T-lymphocyte immunophenotyping

Complete blood counts (CBC) were performed by a standard automated method (Biochem Immunosystems, Allentown, PA) on EDTA anticoagulated blood. CD4 and

CD8 T lymphocyte immunophenotyping was performed by flow cytometry using a two-color whole-blood lysis technique (Q-Prep, Coulter, Hialeah, FL) (Reimann *et al.,* 1994). Fifty microliters of whole blood was incubated in the dark at 25°C with anti-CD4 (Leu3a, Becton Dickinson, Mountain View, CA) and anti-CD8 (Leu2a, Becton Dickinson, Mountain View, CA)-specific monoclonal antibodies according to manufacturer's instructions and were analyzed by flow cytometry using a FACSCAN (Becton Dickinson, Mountainview, CA).

Detection of virus in tissues

In situ hybridization/immunohistochemistry was performed as previously described (Heise *et al.,* 1993; Mandell *et al.,* 1995). A 9-kb SIVmac239 genomic DNA fragment containing the entire *gag*, *pol*, and *env* regions was radioactively labeled with [^{35}S]CTP by random priming in a DNA polymerase reaction to synthesize a SIV DNA probe with a specific activity of ≥ 1 [mult] 10^8 cpm/μg. To detect both SIV DNA and RNA, slides with coverslips were heated at 95°C for 7 min, cooled on ice for 3 min, then incubated overnight at 37°C in humidification chambers. *In situ* hybridization experiments were repeated at least three times to verify consistency of results. Control samples included 4% paraformaldehyde fixed SIV-infected and uninfected cultured CEMx174 cells, hybridization of lymph node and gastrointestinal tissue from SIV-infected and uninfected monkeys, hybridization with probe containing only the pSP64 vector, and RNase treatment of tissue before hybridization. Monocyte/macrophages and T lymphocytes were localized, respectively, using a monoclonal antibody specific for macrophages (HAM-56, DAKO Corporation, Carpentieria, CA) and a polyclonal antibody specific for CD3$^+$ T lymphocytes (CD3, DAKO). These antibodies have been validated for use on uninfected and infected rhesus macaque tissues (Mandell *et al.,* 1995).

Polymerase chain reaction (PCR) and sequencing of viral DNA

For PCR amplification of viral DNA, a forward primer to conserved SIVmac239 sequence (6631nt) and a reverse primer 3′ of the HIV-1-SF33 env SU/TM cleavage site (2157nt) were used. Nucleotide (nt) positions are from the sequence of SIVmac239 clone (GenBank Accession No. M33262) and HIVSF33 (GenBank Accession No. M38427), respectively. Oligonucleotide primers were designed with Amplify v1.0 (Bill Engels, University of Wisconsin, Madison, WI). The forward primer is SHIV115.24 or SHIV121.26 (5′CGGATGCATCCACTCCAGAATCGG or 5′GGCGGATGCATCCACTCCAGAATCGG) and the reverse primer is SHIV116.24 or SHIV122.25 (5′TGTTGCGCCTCAATAGCTCTCAGC or 5′GTGTTGCGCCTCAATAGCTCTCAGC). The sequence of primers SHIV121 and SHIV122 is the sequence of primers SHIV115 and SHIV116 with G and GG added, respectively, to the 5′ end. This 5′ G addition increases the terminal transferase activity of *Taq* DNA polymerase to add A to the 3′ end, thereby increasing cloning efficiency with DNA synthesized with thermophilic polymerases. Template for amplification was prepared from CEMx174 cells infected with SHIV33A virus stock (26,303 $TCID_{50}$/ml) and lysed at Day 7 p.i. PCR was done with 0.5–2 μl of lysate in a final volume of 50 μl of the following reaction mix: 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each dATP, dCTP, dGTP, and dTTP; 20–40 pmol of each primer; and 1–1.5 units AmpliTaq DNA polymerase (Perkin-Elmir Cetus), overlayed with 40 μl of mineral oil. This reaction mix was transferred to a DNA thermal cycler (Perkin–Elmer Cetus), one cycle at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 or 3 min followed by 20–90 min at 72°C incubation. The 2170-nt PCR product for clones 44.1 and 44.9 was Qiaex (Qiagen, Valencia, CA) purified from agarose gel electrophoresis of the PCR reaction and ligated to pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, CA). For clones 52.12, 52.13, and 52.15, 3 μl of the PCR reaction was directly ligated to pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, CA); and for clone 53.10, the PCR reaction was processed with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and ligated to the pCR2.1 using the TA cloning kit. Plasmid clones were screened for insert size and sequenced. Amino acid alignment was determined using GeneWorks (Oxford Molecular, Campbell, CA). The DNA sequences of these clones are deposited in GenBank and will be available simultaneous with publication of this report.

Plasmid vectors for *env* gene expression and cell fusion assay

The gp160 env gene of SHIV$_{SF33}$ and the two clones from SHIV$_{SF33A}$ were inserted into the mammalian cell expression vector pND-14, which contains the cytomegalovirus immediate early promoter and intron-A, the constitutive transport element of the simian type-D retrovirus-1, and the poly(A) signal from bovine growth hormone (G. Rhodes and P. Luciw, unpublished results). This vector also contains the signal leader sequence of tissue plasminogen activator (TPA) followed by an oligonucleotide polylinker with several unique restriction enzyme sites for cloning (Chapman *et al.,* 1991). PCR amplified *env* gene DNA fragments were cloned into the polylinker so that the TPA signal leader substituted for the *env* gene leader. pND-33 contains the HIV-1$_{SF33}$ *env* gp160 gene. The *Kpn*I to *Mun*I DNA fragment, containing most of the gp120 sequences, was prepared from SHIV$_{SF33A}$ env clone 44.9 and clone 52.15 (Fig. 6) and used to replace the counterpart region in pND-33 to produce pND-33A1 and pND-33A2, respectively. For immunoblot analysis, a 35-mm plate of BHK-21 cells (3×10^5) was transfected with 4 μg of each plasmid expression vector in a 1–2 M complex of plasmid DNA (4 μg per plate) and cationic

lipids (1:1 ratio of DOTAP: DOPE; Avanti Polar Lipids, Birmingham, AL). At 48 h after transfection, the culture medium was removed, the cell surface was washed with phosphate-buffered saline, and 0.2 ml of gel disruption buffer (containing 0.4% SDS) was added. Goat polyclonal antibody to HIV-1-SF2 *env* gp120 was obtained from Dr. S. Barnett (Chiron Corporation, Emeryville, CA). Details of acrylamide gel electrophoresis and immunoblotting have been previously described (Sawai *et al.,* 1994). For the fusion assay, BHK-21 cells were grown in a 24-well microtiter plate, at 6×10^4 cells per well. Each well was transfected as described above but with 1 μg of plasmid DNA per well. Quadripulicate wells were transfected with each plasmid vector. At 48 h after transfection, the culture medium (1 ml) was replaced with fresh medium containing 5×10^5 CEMx174 or PM-1 cells. After nine additional hours of incubation, individual wells were observed under a light microscope ([mult]100), and multinucleate syncytia were counted by identifying large cells containing four or more nuclei per cell. Syncytia in a total of six fields per well were counted (four wells per vector), and the average number of syncytia was calculated for each plasmid vector.

ACKNOWLEDGMENTS

We thank Ms. Claudia Jo Weber for expert technical assistance in various aspects of this project. Drs. Ross Tarara, Don Canfield, and Murray Gardner are gratefully acknowledged for performing the pathology studies and assisting with the histopathology analysis; Jo-Anne Yee is thanked for performing the ELISAs to measure antiviral antibody titers. This research was supported in part by National Institutes of Health Grants AI-41907 to P.A. L., AI-41945 to C. C-M., AI-01234 to C.P.M., and RR-00169 to the Base Grant to the California Regional Primate Research Center.

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