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Persistent Infection of Macaques with Simian-Human Immunodeficiency Viruses

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Chimeric simian-human immunodeficiency viruses (SHIV) containing the human immunodeficiency virus type 1 (HIV-1) *tat*, *rev*, *env*, and, in some cases, *vpu* genes were inoculated into eight cynomolgus monkeys. Viruses could be consistently recovered from the CD8-depleted peripheral blood lymphocytes of all eight animals for at least 2 months. After this time, virus isolation varied among the animals, with viruses continuing to be isolated from some animals beyond 600 days after inoculation. The level of viral RNA in plasma during acute infection and the frequency of virus isolation after the initial 2-month period were higher for the Vpu-positive viruses. All of the animals remained clinically healthy, and the absolute numbers of CD4-positive lymphocytes were stable. Antibodies capable of neutralizing HIV-1 were generated at high titers in animals exhibiting the greatest consistency of virus isolation. Strain-specific HIV-1-neutralizing antibodies were initially elicited, and then more broadly neutralizing antibodies were elicited. *env* sequences from two viruses isolated more than a year after infection were analyzed. In the Vpu-negative SHIV, for which virus loads were lower, a small amount of *env* variation, which did not correspond to that found in natural HIV-1 variants, was observed. By contrast, in the Vpu-positive virus, which was consistently isolated from the host animal, extensive variation of the envelope glycoproteins in the defined variable gp120 regions was observed. Escape from neutralization by CD4 binding site monoclonal antibodies was observed for the viruses with the latter envelope glycoproteins, and the mechanism of escape appears to involve decreased binding of the antibody to the monomeric gp120 glycoproteins. The consistency with which SHIV infection of cynomolgus monkeys is initiated and the similarities in the neutralizing antibody response to SHIV and HIV-1 support the utility of this model system for the study of HIV-1 prophylaxis.

Human immunodeficiency virus types 1 and 2 (HIV-1 and -2) are etiologic agents of AIDS in humans (3, 7, 12, 15). These viruses are related to the simian immunodeficiency viruses (SIV), which infect a number of monkey species and can induce an AIDS-like disease in macaques (10, 11). The infection of chimpanzees by HIV-1 and the infection of monkeys by SIV have been useful animal models for the study of prophylaxis against the primate immunodeficiency viruses (10, 14). The expense and limited availability of chimpanzees and the differences between HIV-1 and SIV (23) have motivated searches for animal models in which HIV-1-like viruses infect readily available monkey species. HIV-1 has been reported to infect pig-tailed macaques, but the great inoculum size required for these infections may limit the general utility of this model for prophylactic studies (1, 13). Another approach involves chimeric viruses containing HIV-1 and SIV_{mac} genetic information. These chimeric viruses, called simian-human immunodeficiency viruses (SHIV), have been shown to infect monkey peripheral blood mononuclear cells (PBMC) and to initiate the infection of cynomolgus monkeys (29, 35, 37).

In addition to the *gag*, *pol*, and *env* genes typical of retroviruses, the primate immunodeficiency viruses encode several regulatory proteins (21). While functional Tat and Rev proteins are absolutely required for viral replication, the in vitro phenotypic effects associated with the deletion of the *vif*, *vpx*, *vpr*, *vpu*, and *nef* genes are dependent upon the host cell type. The study of SIV variants containing deletions in the *vpr* or *nef* genes from monkeys has revealed in vivo functions in virus replication and pathogenicity, some of which are not evident in vitro (25, 27).

Unlike the *vif*, *vpx*, *vpr*, and *nef* genes, the *vpu* gene is unique in HIV-1 and SIV_{cpz}. The Vpu protein down-regulates the steady-state levels of the CD4 glycoprotein (28, 43, 44), the receptor for the primate immunodeficiency viruses (9, 26). Vpu also facilitates the assembly and release of virion proteins in a cell-type-dependent manner (16, 17, 40, 46). The HIV-1 Vpu protein has been shown to enhance the release of virions from a wide range of retroviruses, including HIV-2, Maedi-Visna virus, and Moloney murine leukemia virus (17). Here we describe the construction of a SHIV capable of encoding the HIV-1 Vpu protein and compare the course of infection and elicitation of humoral immune responses in cynomolgus monkeys with those of the Vpu-negative counterpart.

MATERIALS AND METHODS

Plasmid construction, cells, transfections, and reverse transcriptase assays. The 5′ and 3′ halves of the SHIV proviral clone were contained on two separate plasmids, designated p5′SHIV and p3′SHIV, respectively. The Vpu^- SHIV used in this study corresponds to SHIV-4 in reference 29. The *vpu* sequence in p3′SHIV was modified by site-directed mutagenesis to create the p3′u+SHIV plasmid. The introduced changes were confirmed by DNA sequencing (see Fig. 1).

Ligation of *Sph*I-digested p5′SHIV with either *Sph*I-digested p3′u+SHIV or p3′SHIV resulted in the formation of complete proviral DNA for the Vpu^+ or Vpu^- SHIV, respectively. The ligated DNA was transfected into CEMx174 cells as described previously (29). CEMx174 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Virus production in the supernatants of

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transfected cells was monitored by measurement of reverse transcriptase activity (33). The *Kpn*I-*Bam*HI fragment encompassing *env* from viruses recovered from monkey 357-91 at 461 days post-inoculation (dpi) was cloned into p3′u+SHIV to generate clones A through F. These plasmids were ligated to p5′SHIV and prepared for CEMx174 transfections as described above.

Macaque PBMC were isolated from fresh blood as described previously (29). PBMC were propagated in RPMI 1640 supplemented with 10% fetal bovine serum and 20 U of recombinant human interleukin 2 (Collaborative Research) per ml.

Virus production in transfected or infected cultures was monitored every 3 to 4 days by reverse transcriptase assays as described previously, with 1.5 ml of cell-free supernatant (29). After supernatants for reverse transcriptase assays were removed, cells were resuspended in an amount of fresh medium sufficient to maintain the cell density between 10^5 and 10^6 cells/ml.

Preparation of virus stocks, determination of tissue culture infective doses, and infection of monkeys. The Vpu^+ SHIV was propagated in cynomolgus monkey PBMC and titrated on CEMx174 cells, as was previously described for the Vpu^- SHIV (29). Four cynomolgus monkeys were inoculated intravenously with 3 ml of cell-free supernatant from cynomolgus monkey PBMC containing a total of 7,000 50% tissue culture infective doses of Vpu^+ SHIV.

Virus isolations. Heparinized blood was obtained from the inoculated monkeys on the noted days postinoculation. PBMC were isolated by Ficoll-diatrizoate density gradient centrifugation and stimulated overnight with 6.25 μg of concanavalin A per ml in RPMI 1640 supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 0.02% gentamicin. The cells were washed and $CD8^+$ lymphocytes were removed with the anti-CD8 monoclonal antibody 7PT3F9 (S. Schlossman, Dana-Farber Cancer Institute, Boston, Mass.) and goat anti-mouse immunoglobulin (Ig)-coated magnetic beads (Dynal, Oslo, Norway). The resulting cells were cultivated in interleukin 2-supplemented medium (20 U/ml) at 10^6 cells/ml, and culture supernatants obtained every 3 to 4 days for 18 days were assessed for SIV_{mac} Gag antigen by a commercially available enzyme-linked immunoassay (Coulter). Cultures in which at least a single supernatant had detectable SIV_{mac} Gag antigen are considered positive.

A logistic regression analysis of the virus isolation data was performed with a subroutine for generalized estimating equations (47). The probability of a positive virus isolation was modeled as a decay curve represented as $e^{\alpha + \beta(vpu) + \gamma(1/time)}/[1 + e^{\alpha + \beta(vpu) + \gamma(1/time)}]$, where vpu is either 1 (for Vpu-positive viruses) or 0 (for Vpu-negative viruses) and the time refers to days following inoculation. The coefficients α and γ were −2.49 and 187.4, respectively, with both coefficients having P values of less than 0.0004. α and γ define the parameters of the decay curve, and their significance indicates that the mathematical model we have chosen is appropriate for the data set. The coefficient β represents the contribution of Vpu status to a positive virus isolation, with a β of 0 indicating no effect of Vpu on virus isolation. The estimate of the coefficient β, based on the virus isolation data, was 1.74, with a P value of 0.028.

Quantitative-competitive PCR for measurement of plasma viral RNA. Plasma samples were centrifuged (10,000 × *g*, 15 min, 4°C) to clarify the plasma and remove the platelets. Then, 250 μl of clarified plasma was diluted 1:8 with phosphate-buffered saline (PBS), and virus was pelleted at 40,000 rpm for 70 min in a Beckman type 50.3 rotor (115,200 × g). The virus pellet was resuspended in 300 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], and 1 mg of proteinase K per ml) and incubated at 37°C for 1.5 to 2 h. Samples were extracted three times with phenol-chloroform and once with chloroform. The aqueous phase was adjusted to contain glycogen (40 μg/ml) and a 7.5-kb synthetic RNA (10 ng/ml) (Bethesda Research Laboratories), and RNA was precipitated with ethanol. The RNA was then pelleted and resuspended in 25 μl of water.

The SIV *gag* primers used for quantitative-competitive PCR were 5′-AAAGC CTGTTGGAGAACAAAGAAG-3′ and 5′-AATTTTACCCAGGCATTTA-3′, corresponding to nucleotides 143 to 167 (positive strand) and 479 to 460 (negative strand), respectively, of the *gag* gene of the SIV_{mne} sequence in the Los Alamos database. For cDNA production, 3 μl of the viral RNA preparation was serially diluted (1:4) into buffer containing 500 copies of internal competitor RNA, placental RNase inhibitor (20 U; Boehringer), Moloney murine leukemia virus reverse transcriptase (50 U; Bethesda Research Laboratories), negative-strand primer to a 0.5 μM final concentration, and $MgCl_2$ to a 6 mM final concentration, all in a final volume of 30 μl. Reverse transcription was performed at 42°C for 15 min, and then inactivation was performed at 99°C for 5 min. PCR was performed by adding to each reaction mixture 70 μl of a mix containing 10× PCR buffer (1× after addition to the PCR mixture; Boehringer), *Taq* polymerase (2.5 U; Boehringer) mixed 1:1 with *Taq* Start antibody (Clontech), positive-strand primer to a 0.15 μM final concentration, and $MgCl_2$ to a 3.5 mM final concentration. Thermocycling conditions were 95°C for 1 min and 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 9 min.

Amplified products were separated on 30-well 3% agarose HT gels (ISS Corp.) containing 0.25 μg of ethidium bromide per ml. The internal control PCR product contained an 83-bp deletion which enables discrimination between viral (336-bp) and internal control (253-bp) amplified products. Viral RNA levels were calculated from the dilution of sample, which gives a visual signal equivalent to that of the internal control. For calibration of the assay to absolute numbers of viral particles, SIV tissue culture stocks were counted by transmission electron microscopy and these results were compared with those obtained by quantitative-competitive PCR.

Analysis of DNA of isolated viruses. PBMC culture supernatants containing virus from SHIV-infected animals were passed through a 0.45-μm-pore-size filter and then centrifuged at 120,000 × *g* to pellet the virus. Virus pellets were resuspended in 0.5 ml of lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and extracted three times with an equal volume of 1:1 phenol-chloroform. Genomic RNA was ethanol precipitated and resuspended in water.

Primer P16777 (5′-CATCTTCCACCTCTCCTAAGAGTCTCCC-3′), a negative-strand primer downstream of the *Rsr*II site in the SHIV proviral sequence (30), and Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) were used to initiate cDNA synthesis reaction with 1 μg of genomic RNA. The 20-μl synthesis reaction mixture was diluted to 100 μl with water, and 1 μl was used for first-round PCR. First-round PCR primers were P11744 (5′-CAACCT GGGGGAGGAAATCCTCTCTC-3′), a positive-strand primer upstream of the *Sph*I site in SIV_{mac239} *vpr*, and P16775 (5′-GCCCGCAAGAGTCTCTGTCG CAGATC-3′), a negative-strand primer downstream of the *Rsr*II site in SHIV. The second-round primers were P11745 (5′-GCTATACCGCCCTCTAGAAG CATGC-3′) (*Sph*I) and P16774 (5′-CAGACGGCCTGGACCGCCTCATGG-3′) (*Rsr*II). The resulting PCR product was 2.9 kb in length.

PCR products were agarose-gel purified with Gene Clean II (Bio 101) and sequenced with the *fmol* DNA sequencing system (Promega) and [γ-^{32}P]ATP. Sequencing reactions were run on 6% urea–polyacrylamide gels and visualized by autoradiography. Alternatively, PCR products were digested with *Kpn*I (site in C1 region of gp120) and *Bam*HI (site in the cytoplasmic tail of gp41) and cloned into the p3′u+SHIV vector. Sequencing of clones was conducted with dye-labeled dideoxy terminators, and samples were analyzed on an Applied Biosystems model 373A automated DNA sequencer as described previously (38).

Radioimmunoprecipitation assays. Approximately 10^7 CEMx174 cells chronically infected with SHIV were labeled overnight with [^{35}S]Cys or [^{35}S]Met, were lysed, and were immunoprecipitated with pooled sera from patients with AIDS or a mouse polyclonal anti-Vpu serum (a gift from Eric Cohen, University of Montreal) as described previously (41).

Plasma anti-gp120 and anti-gp160 antibody titers. For anti-gp120 enzyme-linked immunosorbent assay (ELISA) titers, 96-well round-bottom microtiter plates (Costar) were incubated with 100 μl of a solution of HIV-1 IIIB gp120 (Agmed) (1 μg of 100 mM Na_2HCO_3-Na_3CO_3, pH 9.5, per ml) per well overnight at 4°C. The next day, the liquid was removed from the wells and 200 μl of blocking buffer (2% milk powder–5% fetal bovine serum in PBS) was added for 2 h at room temperature. After three washes with 0.1% Tween 20 in PBS, various dilutions of monkey plasma in blocking buffer were added for 1 h at room temperature. After three washes with 0.1% Tween 20 in PBS, peroxidase-conjugated goat anti-monkey IgG serum (1:10,000 dilution in blocking buffer; Sigma Immunochemicals) was added for 30 min at room temperature. After being washed three times, the plates were developed with 3,3′,5,5′-tetramethybenzidine peroxidase enzyme immunoassay substrate (BioRad) and the reaction was stopped with 0.2 N sulfuric acid. The A_{450} was measured. Wells were considered positive if both wells of the duplicate at a given plasma dilution exhibited an optical density greater than 0.6 (for the 1/500 plasma dilution) or greater than 0.275 (for all higher dilutions). These values are three times the mean optical density of the preinfection plasma samples used as negative controls.

For anti-gp160 antibody titers, 5 μl of animal plasma was used to immunoprecipitate [^{35}S]Cys-labeled HXBc2 envelope glycoproteins in lysates of SHIV-infected CEMx174 cells as described previously (41). Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. The gp160 bands were quantitated by densitometry.

Neutralization of recombinant HIV-1 viruses. Recombinant HIV-1 viruses expressing the bacterial chloramphenicol acetyltransferase gene and containing the HXBc2, MN, 89.6, or clone C and clone E (from animal 357-91) envelope glycoproteins were prepared in COS-1 cells as described previously (22). The MN *env* gene was a gift from Phillip Berman (Genentech). The *Kpn*I-*Bsm*I fragment of the MN *env* gene (20) was cloned into the pSVIIIenv expressor plasmid, resulting in a plasmid expressing an envelope glycoprotein with almost the complete gp120 and part of the gp41 ectodomain derived from the MN virus. The 89.6 strain of HIV-1 has been described previously (8). The *Kpn*I-*Bam*HI fragment of the 89.6 *env* gene was cloned into the pSVIIIenv expressor plasmid, resulting in DNA capable of expressing almost the complete gp120 and the whole ectodomain of gp41 from the 89.6 virus. The *Kpn*I-*Bam*HI fragments of *env* clones C and E, derived from animal 357-91 at 461 dpi, were similarly cloned into pSVIIIenv. For most neutralization assays, 100 μl of the recombinant virus produced from transfected COS-1 cells was incubated with 2 μl of monkey plasma for 1 h at 37°C before the addition of Jurkat lymphocytes. In some assays, greater dilutions of monkey plasma were utilized. Chloramphenicol acetyltransferase activities in the target cells were measured 3 days later as described previously (41).

Monomeric gp120 binding assays. The pSVIIIenv plasmids containing the wild-type HXBc2 *env* gene or the clone C and clone E *env* genes were transfected into COS-1 cells to produce soluble monomeric gp120 glycoproteins in the supernatants. Equal volumes of radiolabeled supernatant were precipitated with 1 μg of monoclonal antibodies or an excess of pooled sera from patients with AIDS as described previously (41). The immunoprecipitated gp120 glycoprotein

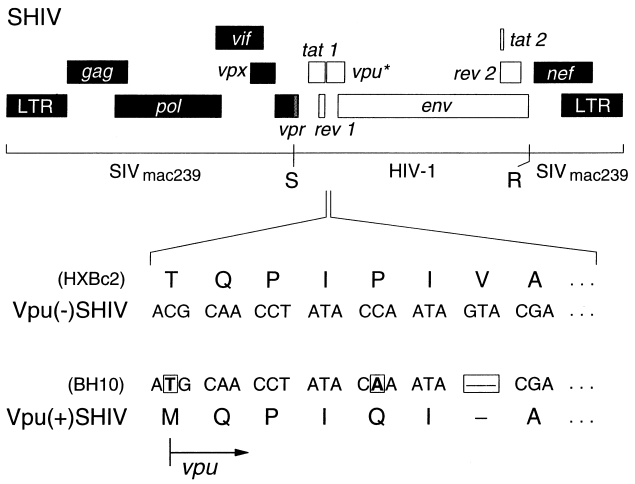

FIG. 1. Structure of the SHIV chimeras and construction of the Vpu$^+$ SHIV. The upper portion of the figure shows the composition of the chimeric SHIV used in this study, with the components derived from SIV_{mac239} in black squares and the HIV-1-specific components in white squares. The 3′ end of the *vpr* gene was reconstituted as a result of the ligation at the *Sph*I (S) site and is stippled in the diagram. The 3′ HIV-1–SIV junction is at the *Rsr*II (R) site. The *vpu* gene is designated with an asterisk. The lower portion of the figure shows the nucleotide and predicted amino acid sequences of the 5′ end of the *vpu* gene. The sequences in the Vpu$^-$ SHIV are similar to those of the HXBc2 HIV-1 variant, while the sequences in the Vpu$^+$ SHIV are similar to those of the BH10 HIV-1 variant.

was subjected to SDS-PAGE and quantitated by densitometry. Envelope expression levels in different COS-1 transfections were controlled for by normalizing all values to the level of gp120 glycoprotein precipitated by serum from patients with AIDS.

RESULTS

Infection of cynomolgus monkeys with Vpu$^+$ and Vpu$^-$ SHIV. To examine the contribution of the HIV-1 Vpu protein to the replication of the SHIV chimera, a Vpu$^+$ SHIV containing a functional *vpu* gene was constructed (Fig. 1). The sequence of the *vpu* gene in the Vpu$^+$ SHIV is identical to that of the BH10 strain of HIV-1, which has been shown to encode a functional Vpu protein (40).

The Vpu$^+$ and Vpu$^-$ SHIV were produced by transfection of proviral clones into CEMx174 cells as previously described (29). Figure 2A shows the production of reverse transcriptase in the supernatants of these cultures at various times after transfection. No significant difference in the replication kinetics of Vpu$^+$ and Vpu$^-$ SHIV in CEMx174 cells was observed. Cell-free infection of cynomolgus monkey PBMC with SHIV derived from the CEMx174 cultures also indicated that the replication rates of the Vpu$^+$ and Vpu$^-$ SHIV viruses were comparable (Fig. 2B).

Four cynomolgus monkeys were intravenously inoculated with 7,000 50% tissue culture infective doses of the Vpu$^-$ SHIV, and another four animals were inoculated with the same dose of the Vpu$^+$ SHIV. All of the cynomolgus monkeys were 1 to 2 years old at the time of inoculation. The animal infections were monitored by the isolation of virus from CD8-positive lymphocyte-depleted, concanavalin A-stimulated PBMC of the monkeys at various times after inoculation. The data in Fig. 3 indicate that all of the monkeys inoculated with either the Vpu$^+$ or Vpu$^-$ SHIV became infected. For the animals infected with the Vpu$^-$ SHIV, virus isolation at >60 dpi was infrequent and intermittent. Nonetheless, viruses could be isolated from two of these animals at >500 dpi. After the initial 2 months of infection, virus isolation from the Vpu$^+$ SHIV-infected monkeys was more frequent than virus isolation from the Vpu$^-$ SHIV-infected animals ($P = 0.028$; see Materials and Methods). For two of the animals inoculated with the Vpu$^+$ SHIV, virus isolation was consistently successful for up to 300 dpi. Even at >850 dpi, Vpu$^+$ SHIV isolation from animal 357-91 was consistently positive (data not shown). All of the animals remained clinically healthy during the course of the study, and no consistent changes in the absolute numbers of CD4- or CD8-positive lymphocytes were observed (data not shown).

Although the frequency of virus isolation is an approximate indicator of virus loads, we sought to directly measure the level of viral RNA in the plasma of SHIV-infected animals. During the acute phase of infection, the level of plasma viremia achieved in Vpu$^+$ SHIV infection was greater than that achieved in Vpu$^-$ SHIV infection, during which viral loads were below the level of detection (Table 1). When later plasma samples were examined, the viral loads from all eight animals were below the level of detection (data not shown). In the Vpu$^+$ SHIV-infected animals, the level of early plasma virus RNA was higher in the animals with the greater frequency of successful virus isolations.

Antibody responses in SHIV-infected animals. To analyze the humoral immune response in SHIV-infected monkeys, plasma samples obtained at different times after infection were used to precipitate ^{35}S-labeled HIV-1 envelope glycoproteins from lysates of SHIV-infected CEMx174 cells. The amount of gp160 glycoprotein precipitated was quantitated by densitometric analysis of autoradiograms of SDS-PAGE gels of the precipitates. By this assay, antibodies capable of specifically precipitating the HIV-1 gp160 glycoprotein were detected in

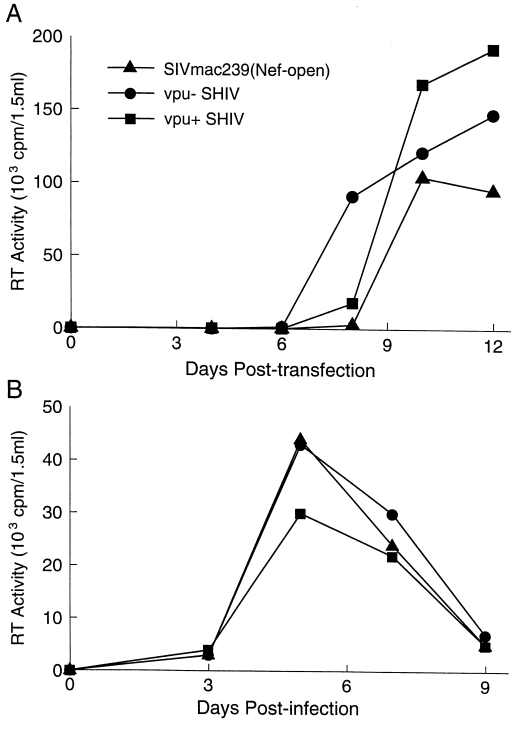

FIG. 2. Replication of Vpu$^+$ and Vpu$^-$ SHIV in vitro. (A) The rates of production of reverse transcriptase in the supernatants of CEMx174 lymphocytes transfected with proviral DNA for the Vpu$^+$ and Vpu$^-$ SHIV are shown. (B) Cynomolgus monkey PBMC were infected with equivalent reverse transcriptase units of cell-free Vpu$^+$ and Vpu$^-$ SHIV, and the reverse transcriptase activities were monitored in the cell supernatants.

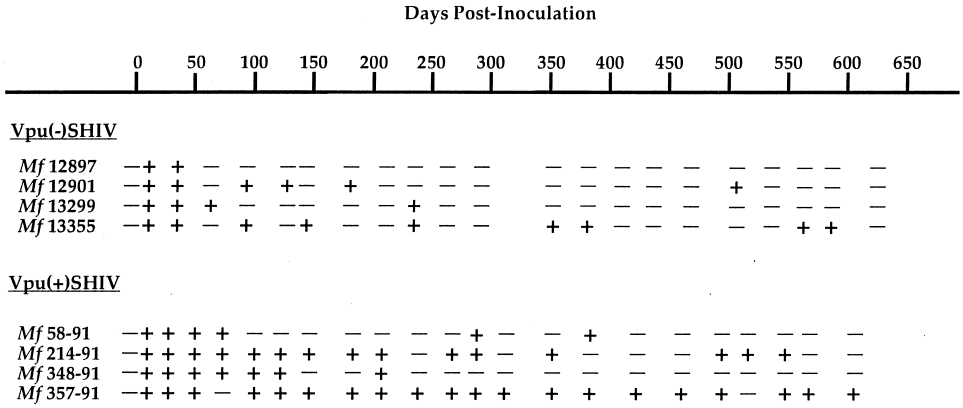

FIG. 3. Isolation of SHIV from the CD8-depleted, ConA-stimulated PBMC of cynomolgus monkeys at various times following inoculation. The first four monkeys listed were infected with Vpu^- SHIV, while the other four animals listed were inoculated with Vpu^+ SHIV.

all infected animals within 2 months of inoculation (Fig. 4 and data not shown). Variability in the anti-gp160 immune response among all eight animals, regardless of the Vpu status of the infecting virus, was seen (Fig. 4). Animals 13355 and 357-91 exhibited the highest anti-gp160 responses.

The immunoprecipitation analysis described above also indicated that all of the inoculated monkeys generated serum antibodies reactive with the HIV-1 gp120 glycoprotein (data not shown). The titers of anti-gp120 antibodies at different times following infection with the Vpu^+ or Vpu^- SHIV were determined by ELISA (Fig. 5). Within each group of animals, a wide range of anti-gp120 antibody titers was observed. As was observed for the anti-gp160 immune response, animals 13355 and 357-91 generated the highest titers of antibodies reactive with the HIV-1 gp120 glycoprotein. With the exception of monkey 357-91, the Vpu^+ SHIV-infected animals exhibited anti-gp120 antibody titers lower than those seen in the Vpu^- SHIV-infected animals (Fig. 5). This may relate to the effect of the *vpu* initiation codon on the level of expression of envelope glycoproteins (36).

SHIV were neutralized in tissue culture assays by monoclonal antibodies directed against the V3 loop or CD4 binding site of the HIV-1 gp120 glycoprotein (data not shown). To determine if SHIV infection results in the elicitation of HIV-1-specific neutralizing antibodies, we used a single-step *env* complementation assay. In this assay, recombinant HIV-1 virions containing the envelope glycoproteins of the HXBc2, MN, or 89.6 HIV-1 strains and encoding the bacterial chloramphenicol acetyltransferase protein were produced in transfected COS-1 cells as described previously (22). Recombinant virions were incubated with plasma derived from the monkeys either prior to infection or at various times following infection. Figure 6 illustrates the neutralizing activity for the homologous HXBc2 virus in infected monkey plasma. The neutralizing activity roughly correlated with the anti-gp120 and anti-gp160 antibody titers. Monkeys 13355 and 357-91 exhibited the highest anti-gp120 titers and the greatest potencies of neutralization against the HXBc2 virus.

Neutralization of the recombinant HIV-1 virions containing

TABLE 1. Plasma viral RNA levels 2 weeks after inoculation with SHIV

vpu status of SHIV	Animal no.	Particles/ml of plasma[a]
Negative	12897	<10,000
	12901	<10,000
	13299	<10,000
	13355	<10,000
Positive	58-91	66,000
	214-91	352,000
	348-91	704,000
	357-91	2,820,000

[a] The limit of detection for this assay is 10,000 particle equivalents per ml.

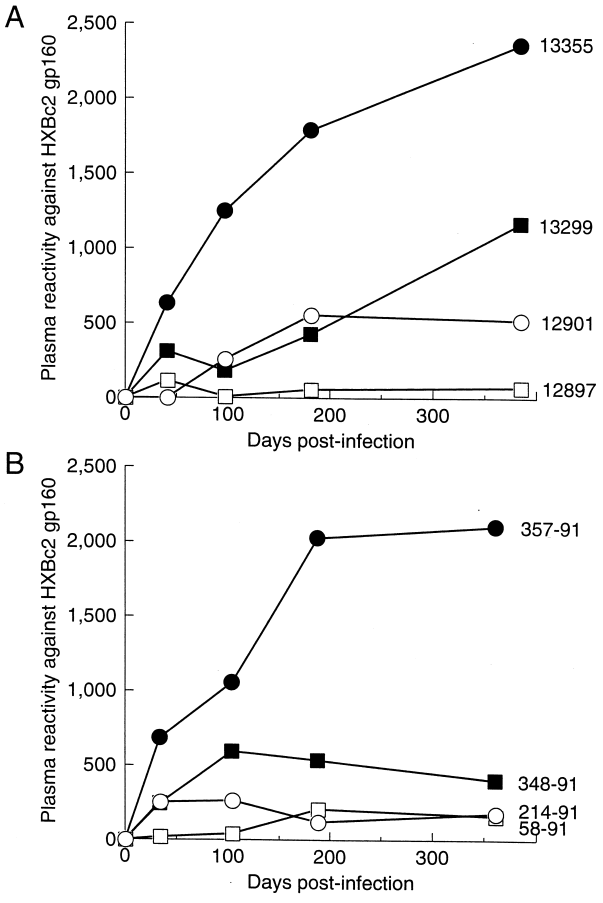

FIG. 4. Anti-HIV-1 gp160 antibody titers in plasma from Vpu^- (A) and Vpu^+ (B) SHIV-infected monkeys. Monkey numbers are shown on the right.

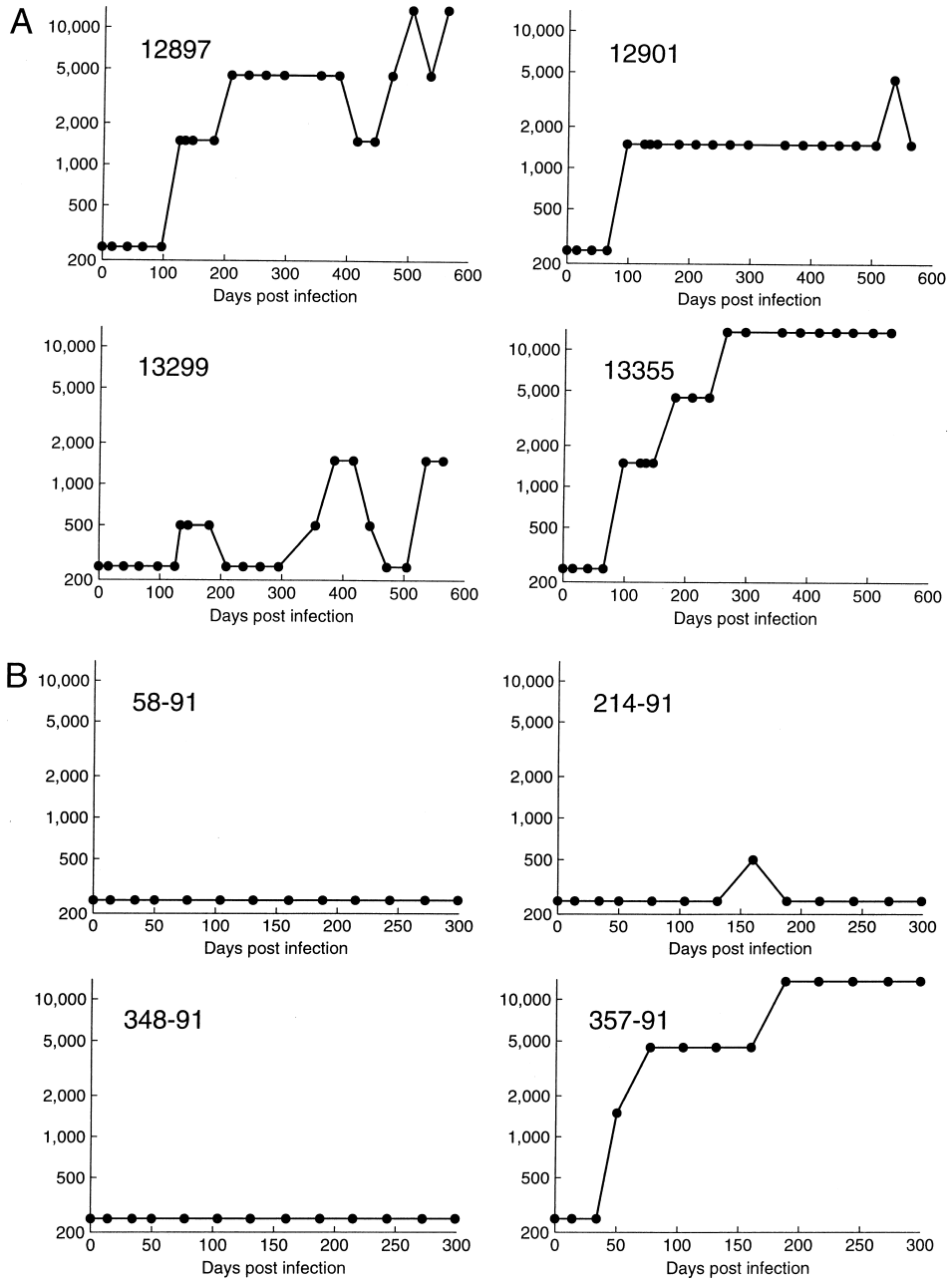

FIG. 5. Anti-HIV-1 gp120 antibody titers in plasma from SHIV-infected cynomolgus monkeys. The titers of anti-HIV-1 gp120 antibodies in the plasma of cynomolgus monkeys infected with Vpu^- (A) and Vpu^+ (B) SHIV are shown.

MN or 89.6 envelope glycoproteins was studied to determine whether a virus with envelope glycoproteins different from those contained on the infecting SHIV could be neutralized by antibodies in the infected animals' plasma. In addition, the 89.6 virus represents a primary clinical isolate that has been shown to be less sensitive to neutralizing antibodies than are laboratory-passaged isolates, like HXBc2 or MN (39). Plasma samples from animals 13355 and 357-91, which exhibited the highest titers of antibodies neutralizing HIV-1 with the homologous HXBc2 envelope glycoproteins, were used in this study. Figure 7 shows that neutralizing activity against the MN virus appeared with a delay compared with the activity against the homologous HXBc2 virus. Viruses with the 89.6 envelope glycoproteins were more resistant to neutralization by plasma from either animal. Titration of the neutralizing activities (Fig. 8) indicated that soon after the appearance of HXBc2 neutralizing activity, the neutralization titer against the HXBc2 virus was greater than that against the MN virus. By 250 to 300 dpi, the neutralization titers directed against HXBc2 and MN viruses were comparable.

Analysis of viruses isolated from SHIV-infected monkeys. Previous studies of the *nef* gene of SIV_{mac239} in rhesus monkeys indicated that in vivo selective pressure can result in the generation of revertants as early as 2 weeks after infection (27). To evaluate whether revertants differing in the potential to express the Vpu protein arose in the SHIV-infected monkeys,

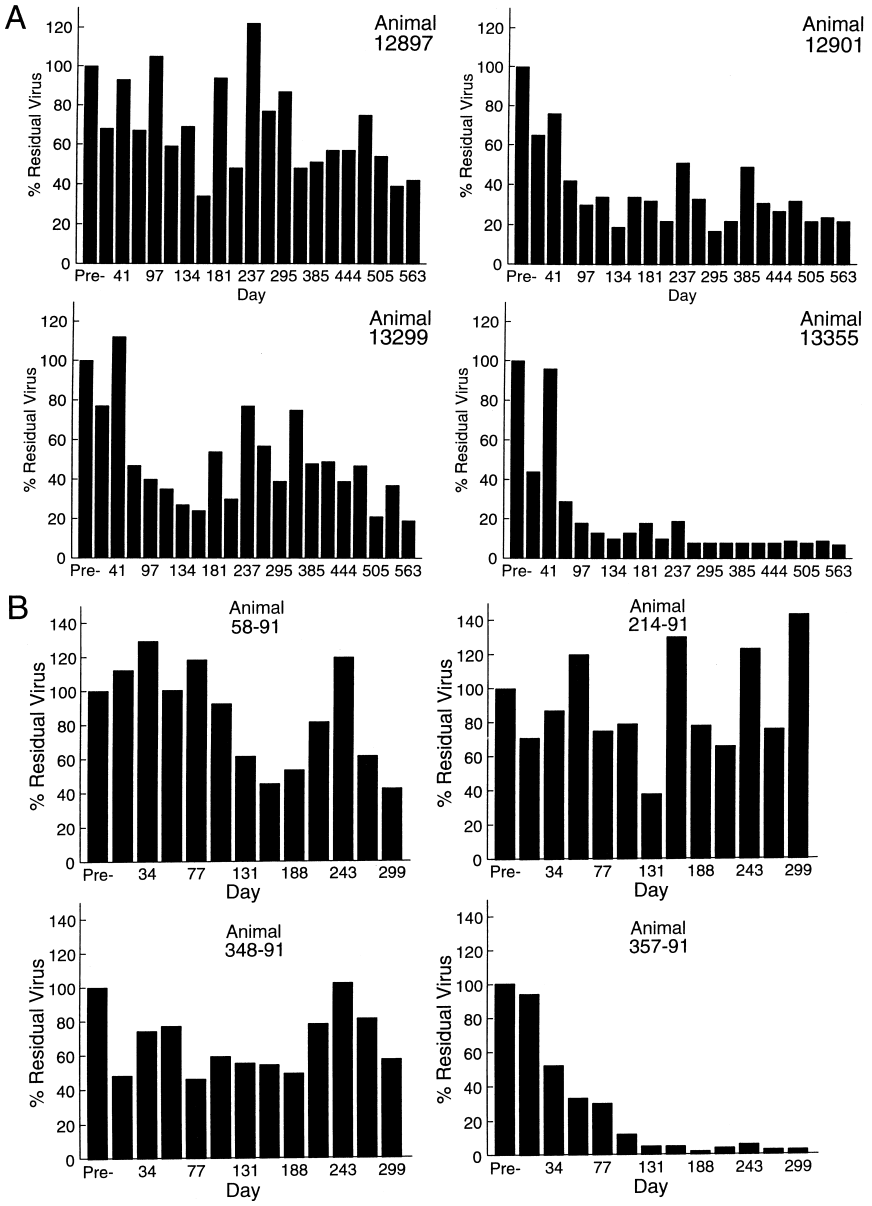

FIG. 6. Neutralization of homologous HIV-1 by plasma from SHIV-infected cynomolgus monkeys. The neutralization of recombinant HIV-1 containing the homologous HXBc2 envelope glycoproteins by a 1:50 dilution of plasma from cynomolgus monkeys infected with Vpu^- (A) or Vpu^+ (B) SHIV is shown. In this assay, 100% residual virus indicates no neutralization, while 0% residual virus indicates complete neutralization.

reisolated viruses were used to prepare viral RNA. Reverse transcription and PCR were used to amplify a 2.9-kb *Sph*I-*Rsr*II fragment encompassing the *vpu* gene. Direct cycle sequencing of the PCR products was used to analyze the status of the *vpu* gene. No reversion of the missense initiation codon in the Vpu^- SHIV viruses isolated at 16 or 41 days after inoculation was observed (data not shown), even though restoration of the HXBc2 *vpu* gene initiation codon is sufficient to allow the expression of a functional Vpu product (28). Viruses recovered from animal 13355 at 385 dpi also demonstrated no reversion to an open *vpu* (data not shown). The sequencing of viruses isolated from animals with Vpu^+ SHIV at 117 or 131 dpi indicated that these viruses retained an intact *vpu* gene (data not shown). Analysis of viruses recovered from animal 357-91 at 461 dpi also indicated that the *vpu* gene remained open (data not shown).

Since direct cycle sequencing of PCR products may fail to detect a reverted subpopulation constituting less than 30% of the heterogeneous population, the production of the Vpu protein by isolated viruses was examined. Figure 9 shows that no Vpu protein was precipitated from lysates of CEMx174 cells infected with viruses derived from Vpu^- SHIV-infected animals. By contrast, the Vpu^+ SHIV produced a clearly detectable Vpu protein. Thus, an analysis of the *vpu* gene and its product did not provide evidence for strong positive or negative selection on the expression of the Vpu protein in SHIV-infected animals.

To determine if SHIV had undergone antigenic variation during the course of infection, the *env* genes of viruses isolated from the Vpu^- SHIV-infected animal 13355 at 385 dpi and the Vpu^+ SHIV-infected animal 357-91 at 461 dpi were sequenced. Direct sequencing of the PCR products from monkey

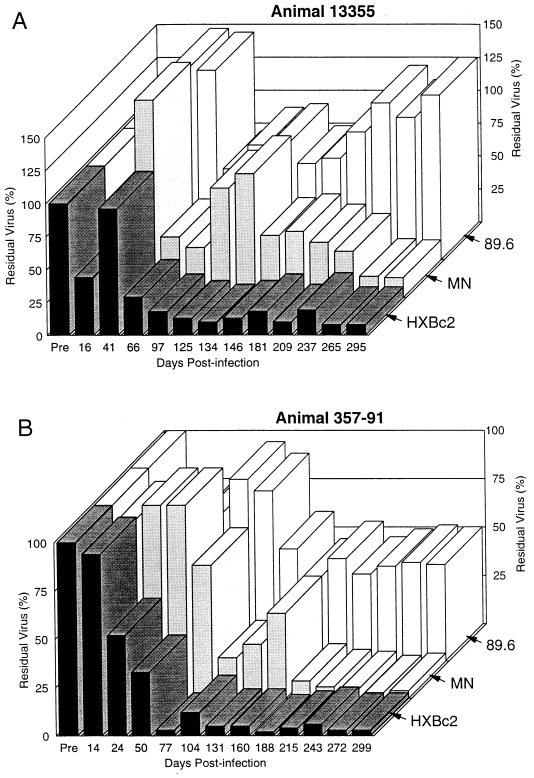

FIG. 7. Comparison of plasma neutralizing activities against HIV-1 with homologous (HXBc2) or heterologous (MN or 89.6) envelope glycoproteins. Dilutions (1:50) of plasma obtained at various times following infection of monkey 13355 with Vpu$^-$ SHIV (A) or of monkey 357-91 with Vpu$^+$ SHIV (B) were used to neutralize envelope glycoproteins as shown.

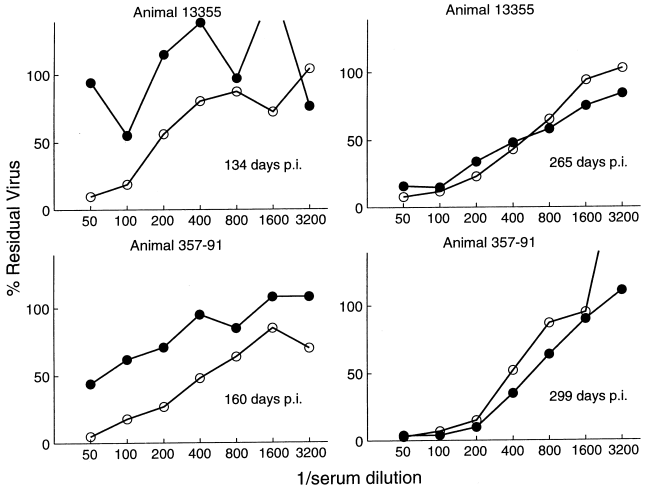

FIG. 8. Titration of HIV-1-neutralizing activities in plasma samples from SHIV-infected monkeys. Plasma from animal 13355, infected with Vpu$^-$ SHIV, and plasma from animal 357-91, infected with Vpu$^+$ SHIV, were tested at various dilutions for abilities to neutralize recombinant HIV-1 containing either the HXBc2 (open circles) or MN (solid circles) envelope glycoproteins. The titrations were carried out with plasma samples from the indicated times postinfection.

13355 indicated a few predominant amino acid changes (Table 2), none of which were in the variable regions of gp120. The *Kpn*I-*Bam*HI fragments (2.1 kb) digested from the *Sph*I-*Rsr*II PCR fragments were cloned into p3′SHIV, and four clones were characterized. Sequencing of these clones from monkey 13355 at 385 dpi confirmed that the predominant mutations found during direct cycle sequencing were also present in the four clones. However, when Vpu$^-$ SHIV containing these envelopes were characterized for replication ability, the four viruses were highly attenuated for growth or did not replicate in CEMx174 cells and macaque PBMC (data not shown).

PCR amplification of the envelope gene and direct sequencing of the PCR products from animal 357-91 at 461 dpi indicated that 14 amino acid changes predominated throughout the envelope gene, and most were concentrated in the variable regions of gp120 (Table 3). *Kpn*I-*Bam*HI fragments digested from the original *Sph*I-*Rsr*II PCR products were cloned into the p3′u+SHIV plasmid for replication studies. Six clones were analyzed for replication competence in CEMx174 cells. Figure 10A illustrates that only clones C and E replicated as efficiently as the parental Vpu$^+$ SHIV after transfection of CEMx174 cells. Cell-free infection of macaque PBMC also indicated no difference in replication abilities of Vpu$^+$ SHIV, clone C, and clone E (Fig. 10B).

The *Kpn*I-*Bam*HI fragments of clones C and E were cloned into the pSVIIIenv envelope expressor plasmid for determining neutralization sensitivity to autologous plasma antibodies from animal 357-91. No evidence for sequential escape from plasma antibodies could be found, although clone E was more resistant to serum-neutralizing antibodies at all time points postinoculation (Fig. 11).

To determine whether the decreased sensitivity of clone E to neutralization by infected animal plasma could be explained by a decrease in sensitivity to particular neutralizing monoclonal antibodies, viruses with wild-type, clone C, or clone E envelope glycoproteins were incubated with anti-CD4 binding site antibodies prior to infection. The IgG12, F105, and 205-43-1 antibodies are directed against the conserved, discontinuous epitopes overlapping the CD4 binding site of the HIV-1 gp120 glycoprotein (30, 32, 34). Compared with the wild-type or clone C envelope glycoproteins, viruses with clone E envelope glycoproteins exhibited resistance to neutralization by monoclonal antibodies F105 and 205-43-1 but were neutralized by IgG12 (Fig. 12). IgG12 has generally exhibited more potent neutralizing activity against both laboratory-passaged and primary HIV-1 isolates than other antibodies directed against the CD4 binding site(s). The resistance to neutralization of clone

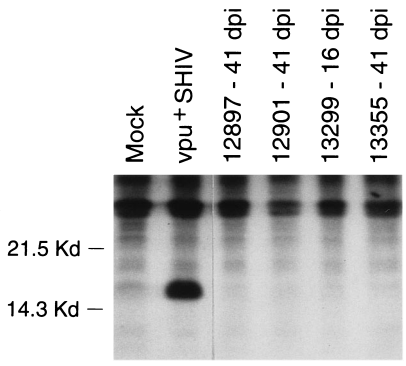

FIG. 9. Precipitation of lysates from CEMx174 cells infected with Vpu$^+$ and Vpu$^-$ SHIV. CEMx174 cells were either mock infected, infected with the Vpu$^+$ SHIV, or infected with SHIV isolated at the indicated times after infection of cynomolgus monkeys with Vpu$^-$ SHIV. The CEMx174 cells were labeled and precipitated with an anti-Vpu serum as described in Materials and Methods.

TABLE 2. Changes in envelope glycoprotein sequences derived from animal 13355 at 385 dpi[a]

Nucleotide no. (change)	Amino acid no. (change)	Region
6396 G/A	58 A/T	C1
6402 G/A	60 A/T	C1
7528 A/C or A/T	435 Y/S or Y/F	C4
8043 G/A	607 A/T	gp41[b]
8101 C/T	626 T/M	gp41[b]

[a] Each nucleotide change is reported as the proviral sequence number, according to the sequence of Muesing et al. (31), with the original base on the left of the shill and the base found in the late virus on the right. The predicted change in amino acid is also shown, with the original residue on the left of the shill and the altered residue on the right. Changes for this animal were observed in the bulk population and were confirmed in all four *env* clones examined.

[b] The altered amino acid was found in the exterior domain of this glycoprotein region.

E is at least partially explained by decreased binding of the monoclonal antibodies to the monomeric gp120 glycoproteins of clone E (Fig. 13). Interestingly, the loss of monomeric envelope recognition by neutralizing monoclonal antibodies is one mechanism by which natural variants of SIV_{mac} escape neutralization by monoclonal antibodies (6).

TABLE 3. Changes in envelope glycoprotein sequences derived from animal 357-91 at 461 dpi[a]

Nucleotide no. (change)	Amino acid no. (change)	Region	Change observed in[b]:		
			Bulk	Clone C	Clone E
5877 G/A	Silent		X	NS	NS
5922 T/C	Silent		X	NS	NS
6238 A/G	146 R/G	V1	X	X	X
6249 G/A	149 M/I	V1	X	X	X
6253 A/C	151 K/Q	V1			X
6284 T/C	161 I/T	V2	X	X	X
6313 A/G	171 K/E	V2	X	X	X
6634 A/G	278 T/A	C2	X	X	X
6700-01 A/G-A/G	300 N/G	V3		X	
6701 A/G	300 N/S	V3	X		X
6748 G/A	316 A/T	V3	X	X	X
6809 C/T	336 A/V	C3	X	X	X
6853 G/A	351 E/K	C3	X	X	X
7033 A/G	411 S/G	V4	X		X
7068 A/G	Silent			X	
7088 A/G	429 K/R	C4	X		X
7104 G/A	434 M/I	C4		X	
7112 C/A	437 P/H	C4		X	
7158 G/A	Silent		X	X	X
7183 A/G	461 S/G	V5	X	X	X
7284 A/G	Silent		X	X	X
7323 G/A	Silent			X	X
7401 C/G	Silent			X	X
7428 A/G	Silent				X
7479 T/A or T/C	Silent		X		
7679 C/T	626 T/M	gp41 (ext.)	X	X	X
7743 A/C	647 E/D	gp41 (ext.)	X	X	
7785 G/A	Silent		X		X
7888 A/G	696 R/G	gp41 (TM)		X	X
7917 G/A	Silent		X	X	X
7923 G/A	Silent		X		X
8020 A/G	740 R/G	gp41 (cyt.)			X
8326-27 C/T-A/T	842 H/I	gp41 (cyt.)	X	NS	NS

[a] Each nucleotide change is reported as the proviral sequence number, according to the sequence of Muesing et al. (31), with the original base on the left of the shill and the base found in the late virus on the right. The predicted change in amino acid is also shown, with the original residue on the left of the shill and the altered residue on the right. The region of the gp120 or gp41 glycoprotein in which the altered amino acid is found is also noted: ext., exterior domain; TM, transmembrane region; cyt., cytoplasmic tail.

[b] X indicates a change that was observed in direct cycle sequencing of a PCR-derived fragment spanning *env* (Bulk) or observed in sequencing a plasmid containing the clone C or clone E *env* fragment. NS, not sequenced.

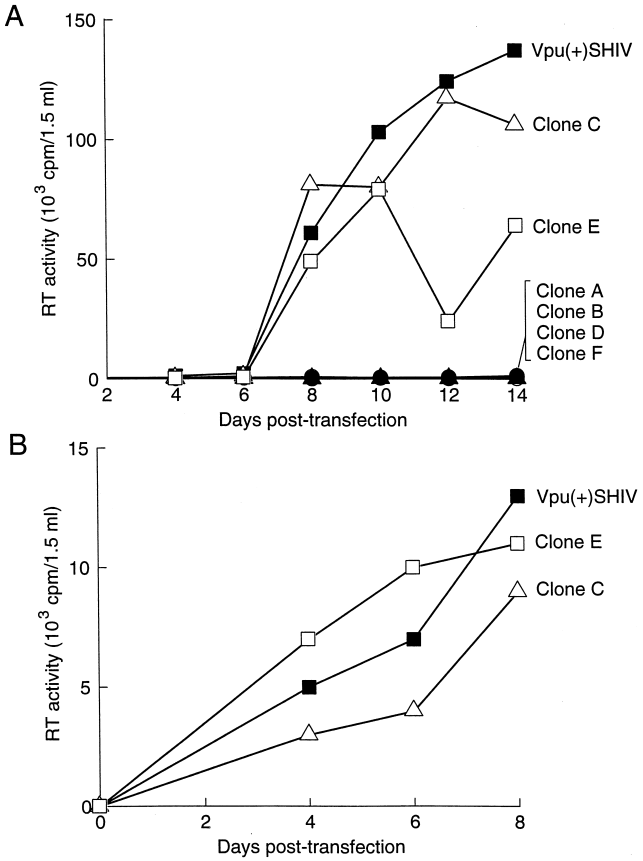

FIG. 10. Replication of clones from monkey 357-91 at 461 dpi. (A) Proviral DNA was transfected into CEMx174 cells, and the level of supernatant reverse transcriptase activity after each transfection is shown. (B) A total of 5,000 reverse transcriptase units of CEMx174-grown Vpu^+ SHIV, clone C, or clone E was used to initiate infection of rhesus macaque PBMC. The level of reverse transcriptase activity in the culture supernatant after each infection is shown.

DISCUSSION

This study demonstrates that SHIV chimeras can consistently initiate infection of cynomolgus monkeys. In all of the infected animals, virus could be recovered for at least 2 months following inoculation. Consistency in initiating infection is one prerequisite for an animal model useful for the study of prophylaxis. It has been reported that virus could be recovered from only one of two cynomolgus monkeys inoculated with a SHIV lacking the *vpr* and *nef* genes (35). The integrity of the SIV_{mac} *nef* gene has been shown to be important for the maintenance of high virus loads in monkeys (25, 27). The abilities of the SHIV chimeras used in this study to encode functional Nef proteins probably contribute to the observed consistency in initiating in vivo infection.

We also studied the course of infection of cynomolgus monkeys by SHIV chimeras differing in the ability to express the HIV-1 Vpu protein. In tissue culture systems, expression of the HIV-1 Vpu protein leads to specific degradation of CD4 (28, 43, 44), the receptor for the primate immunodeficiency viruses (9, 26). Since the HIV-1 Vpu protein can also facilitate the budding of a wide range of retroviruses (17), both defined in vitro functions of the Vpu proteins could potentially modulate

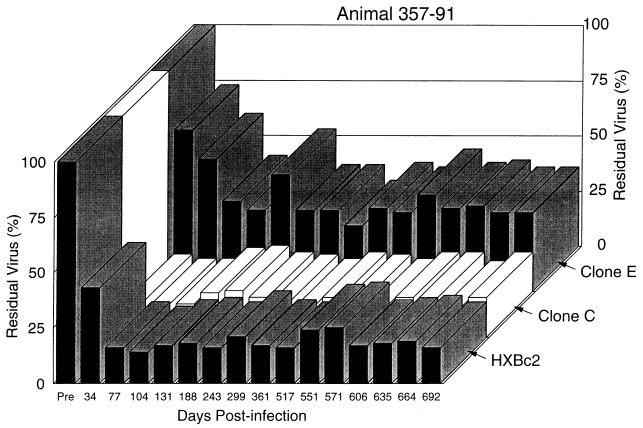

FIG. 11. Comparison of plasma-neutralizing activities against HIV-1 with wild-type HXBc2 envelope glycoproteins or in vivo-adapted envelope glycoproteins (clones C and E) from monkey 357-91 at 461 dpi. Dilutions (1:50) of plasma from various times following infection of monkey 357-91 were used to neutralize HIV-1 with envelope glycoproteins as shown.

the replication of SHIV. Both the virus isolation results and the plasma virus load data early in infection suggest that Vpu acts to increase virus replication in vivo. Given these results, it is surprising that viruses isolated from Vpu$^-$ SHIV-infected animals did not exhibit reversion, allowing a functional Vpu protein to be encoded. It is possible that the in vivo replication of Vpu$^-$ SHIV occurs at such low levels that selective pressures are not always manifest as sequence changes in viruses isolated from the peripheral blood. It is noteworthy that, despite the presence of high titers of neutralizing antibodies in monkey 13355, viruses isolated from that animal after a year postinoculation did not exhibit the expected *env* variation (see below). Perhaps in the case of extremely low levels of in vivo replication, like that of the Vpu$^-$ SHIV, virus isolates arise from cells in which the replicative cycle is latent rather than continuous. In this case, isolated viruses would not necessarily be optimally adapted to selection conditions operative at the time of virus isolation. The low-level replicative ability associated with the envelope glycoproteins of the late Vpu$^-$ SHIV isolates from animal 13355 is consistent with this explanation. A more remote possibility is that the observed differences in replication between Vpu$^+$ and Vpu$^-$ SHIV, although statistically significant, result from a variable other than the presence of an intact *vpu* gene. Undetected differences in the amount of virus inoculated are not likely to account for the results. Titration studies with SHIV indicate that, for inoculum sizes greater than the animal infectious dose, quantitative virus isolations were not affected by a 1,000-fold difference in inoculum size (45). Further studies with SHIV variants that achieve higher levels of in vivo replication will be useful in corroborating the contribution of Vpu to virus load.

Considerable variation in the frequency of virus isolation and the titers of anti-envelope glycoprotein antibodies, including neutralizing antibodies, was observed within the group of four monkeys inoculated with either SHIV variant. This variation presumably results from host-dependent parameters that modulated the overall virus load achieved in the individual animal. These parameters probably do not include the generation of neutralizing antibodies, since the titers of neutralizing antibodies were highest in the animals that exhibited the highest frequency of positive virus isolations in the two groups (13355 and 357-91). An early preference for neutralization of HIV-1 with homologous envelope glycoproteins, followed by a broader response capable of neutralizing viruses with the MN envelope glycoproteins, was observed for these two animals. This temporal pattern of elicitation of neutralizing antibodies has been seen with HIV-1-infected humans and chimpanzees (2, 18, 19, 42).

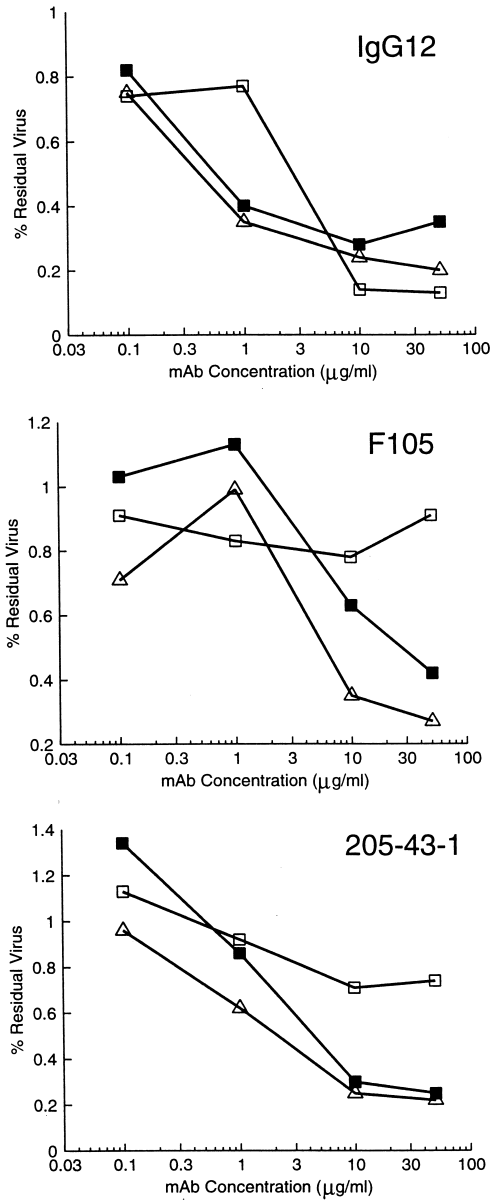

FIG. 12. Neutralization by CD4bs monoclonal antibodies of HIV-1 containing the envelope glycoproteins from HXBc2 (■) or clone C (△) or E (□) from monkey 357-91 at 461 dpi.

We sought to obtain evidence for antigenic variation and neutralization escape in monkey 13355 at 385 dpi with Vpu$^-$ SHIV and in monkey 357-91 at 461 dpi with Vpu$^+$ SHIV. Because of the poor replication ability of virus clones from monkey 13355, neutralization assays for these clones were difficult to perform. Further, the amount of envelope glycoprotein variation seen for viruses from monkey 13355 at 385 dpi was small, and the observed variation was not located in the recognized variable regions of gp120. The minimal envelope glycoprotein variation in the face of high titers of neutralizing

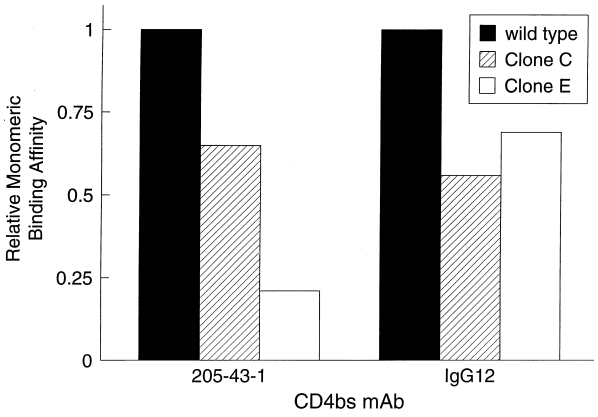

FIG. 13. Monomeric gp120 binding by monoclonal antibodies directed against the CD4 binding site of gp120. Wild-type, clone C, or clone E pSVIIIenv expressors were transfected into COS-1 cells to obtain soluble, monomeric gp120. Equal amounts of COS-1 supernatants were precipitated with monoclonal antibody 205-43-1, IgG12, or pooled sera from AIDS patients. All values were normalized to the amount of gp120 precipitated by patient serum to control for envelope glycoprotein expression in COS-1 cells.

antibodies is paradoxical and may be a result of the low level of in vivo replication associated with the Vpu^- SHIV. In addition to the factors already discussed, selection pressure exerted by neutralizing antibodies may be operative only above a threshold level of virus replication. It has been previously reported that neutralization escape variants of primate lentiviruses emerge in pathogenic but not nonpathogenic infections (4, 24, 48), which are known to differ in the levels of virus replication achieved in vivo. The differences in *env* variation between late virus isolates of Vpu^- and Vpu^+ SHIV may be the result of the lower virus loads achieved in animal 13355 compared with those in animal 357-91.

In our analysis of envelope sequences derived from animal 357-91 at 461 dpi, escape from autologous neutralizing antibodies is at best partial. Escape from some neutralizing monoclonal antibodies can be demonstrated in some clones. Since the nucleotide changes observed in the bulk population and in clones were predominantly missense rather than silent mutations, resulting in amino acid changes concentrated in the variable regions of gp120, immune selection pressures on the envelope glycoproteins are the likely sources of the observed changes. However, complete escape from autologous neutralizing antibodies may not be possible or even necessary for a virus with the degree of replicative capacity exhibited by SHIV in these animals.

Even for the Vpu^+ SHIV, the levels of chronic virus replication are lower than those observed in pathogenic SIV_{mac} infections. Future studies will attempt to identify SHIV chimeras with increased in vivo replicative ability, which is likely to be a prerequisite for pathogenicity in this model system.

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