

## Simian Immunodeficiency Virus Mutants Resistant to Serum Neutralization Arise during Persistent Infection of Rhesus Monkeys

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We previously described the pattern of sequence variation in gp120 following persistent infection of rhesus monkeys with the pathogenic simian immunodeficiency virus SIVmac239 molecular clone (D. P. W. Burns and R. C. Desrosiers, *J. Virol.* 65:1843, 1991). Sequence changes were confined largely to five variable regions (V1 to V5), four of which correspond to human immunodeficiency virus type 1 (HIV-1) gp120 variable regions. Remarkably, 182 of 186 nucleotide substitutions that were documented in these variable regions resulted in amino acid changes. This is an extremely nonrandom pattern, which suggests selective pressure driving amino acid changes in discrete variable domains. In the present study, we investigated whether neutralizing-antibody responses are one selective force responsible at least in part for the observed pattern of sequence variation. Variant *env* sequences called 1-12 and 8-22 obtained 69 and 93 weeks after infection of a rhesus monkey with cloned SIVmac239 were recombined into the parental SIVmac239 genome, and variant viruses were generated by transfection of cultured cells with cloned DNA. The 1-12 and 8-22 recombinants differ from the parental SIVmac239 at 18 amino acid positions in gp120 and at 5 and 10 amino acid positions, respectively, in gp41. Sequential sera from the monkey infected with cloned SIVmac239 from which the 1-12 and 8-22 variants were isolated showed much higher neutralizing antibody titers to cloned SIVmac239 than to the cloned 1-12 and 8-22 variants. For example, at 55 weeks postinfection the neutralizing antibody titer against SIVmac239 was 640 while those to the variant viruses were 40 and less than 20. Two other rhesus monkeys infected with cloned SIVmac239 showed a similar pattern. Rhesus monkeys were also experimentally infected with the cloned variants so that the type-specific nature of the neutralizing antibody responses could be verified. Indeed, each of these monkeys showed neutralizing-antibody responses of much higher titer to the homologous variant used for infection. These experiments unambiguously demonstrate that SIV mutants resistant to serum neutralization arise during the course of persistent infection of rhesus monkeys.

Simian immunodeficiency virus (SIV) is a member of the lentivirus subfamily of retroviruses. This group also includes human immunodeficiency virus (HIV), equine infectious anemia virus (EIAV), visna virus, and caprine arthritis-encephalitis virus. Members of the lentivirus subfamily establish long-term, persistent infections resulting in chronic, nononcogenic, debilitating disease.

Antigenic variation during persistent infection has been documented in at least three lentivirus systems, EIAV (22, 23, 32, 41, 42, 47), visna virus (9, 27, 36-39, 43, 50), and caprine arthritis-encephalitis virus (10, 40). The strongest evidence for immune selection lies in the EIAV system, in which disease is characterized by recurrent clinical episodes of fever, hemolytic anemia, bone marrow depression, lymphoproliferation, immune-complex glomerulonephritis, and persistent viremia (32). Plasma recovered from an infected animal can effectively neutralize virus isolated from earlier febrile episodes but cannot neutralize virus isolated during subsequent febrile episodes (23, 32, 47). The animals in these

studies thus exhibited delayed neutralizing-antibody responses against variant viruses. By inoculating horses with variant strains of EIAV, type-specific neutralizing-antibody responses have been generated against variant viruses (23). Sequence changes responsible for the resistance of variants to serum neutralization have not been defined.

The emergence of neutralization resistant HIV-1 variants in HIV-1 infected chimpanzees (35) and in an HIV-1 infected human (51) has been reported. Sera for neutralization tests were derived from the individual in which the variants emerged. These experiments were performed in one direction only since additional sera from individuals naturally or experimentally infected with variant virus were not available for reciprocal testing of neutralizing activity. This is problematic since different virus stocks show considerable variation in the efficiency with which they score in neutralization tests (25, 28). Such inherent differences in the efficiencies with which virus stocks score in neutralization tests can be due to a variety of factors, including differences in infectivity titers, methods of stock preparation, replication rate, particle/infectivity ratios, gp120 packing density, gp120 structure, gp120-gp41 affinity, gp120-CD4 affinity, design of the assay, and other ill-defined parameters. It has been noted previously that some strains of HIV-1 (e.g., RF) appear considerably resistant to neutralization, while others (e.g., SF2) are considerably more sensitive to neutralization (2, 28, 54).

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Thus, the significance of poor neutralization of variant virus in these previous experiments with HIV-1 (35, 51) is not entirely clear, since it was not possible to demonstrate reciprocal effects with reciprocal sera.

In four other studies on immune selection in humans, HIV-1 isolates were obtained which could not be neutralized by the patients' own sera, indicating that neutralization-resistant mutants had emerged in these individuals (1, 13, 31, 53). In two of these studies (1, 53), the neutralization assay which was used has recently been called into question. The assay, which was based on the detection of viral p24<sup>gag</sup> protein, did not control for anti-gag antibodies which may have been present in the patients' sera. Such anti-gag antibodies can interfere with the detection of viral p24<sup>gag</sup> protein and can result in unreliable neutralization titers (5). In the other two studies (13, 31), variant viruses were not tested against control sera to determine to what extent they were capable of being neutralized. Also, sera from individuals infected with variant virus were not available for reciprocal testing. Therefore, it is difficult to conclude from any of the four studies whether mutants specifically resistant to neutralization by the patient's sera had actually emerged in these HIV-1-infected individuals.

Rhesus monkeys infected with molecularly cloned SIV mac239 provide a useful model system for studying immune selection. More than 50% of rhesus monkeys infected with this cloned virus develop strong antibody responses to the virus and become persistently infected for 1 year or more prior to the development of AIDS (18). By cloning viral envelope genes from rhesus monkeys over time and obtaining sequential serum samples from them, we have been able to study not only the evolution of envelope sequences but also the emergence of neutralization-resistant variants. We have also been able to infect naive monkeys with molecularly cloned variant viruses and to verify the selectivity of the neutralizing-antibody responses. In our previous study on envelope sequence variation, we found that amino acid changes in discrete segments of gp120 result from selective forces operating *in vivo* (4). Results from our present study provide strong evidence that the host neutralizing-antibody response is one of the selective forces driving sequence change in the SIV envelope and that at least some envelope variants are indeed neutralization escape mutants.

## MATERIALS AND METHODS

**Virus.** The SIVmac239 pathogenic molecular clone and its complete sequence have been described previously (18, 19, 34, 45). Molecular clones of *env* variants T69BL1-12 (variant 1-12) and T93V8-22 (variant 8-22) have also been described previously (4). The envelope clone pT69BL1-12 was derived directly from blood of rhesus monkey Mm243-86 at 69 weeks after initial infection with SIVmac239. The envelope clone pT93V8-22 was obtained from cells infected with SIV recovered from the same animal 93 weeks after initial infection. Both *env* clones contain sequence changes representative of the more than 15 *env* clones sequenced from this animal.

**Cells and cell lines.** Continuously growing human CD4<sup>+</sup> cell lines HuT 78 (11), CEMx174 (14), and MT4 (30) were grown in RPMI 1640 medium plus 10% inactivated fetal calf serum, glutamine, penicillin, and streptomycin (complete RPMI). MRC-5 cells (ATCC CCL171; American Type Culture Collection, Rockville, Md.) were grown in basal medium Eagle (BME) (B2900; Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% fetal bovine serum (not inac-

tivated), glutamine, penicillin, and streptomycin (complete BME).

**Experimental infection of rhesus monkeys.** Two juvenile rhesus macaques (*Macaca mulatta*) previously negative for SIV antibody were selected from the New England Regional Primate Research Center colony. Mm206-89 and Mm262-89 were inoculated intramuscularly on 23 January 1991 with virus derived from transfection of cloned viral DNA into CEMx174 cells. Each of the two animals was inoculated with virus containing 7 ng of p27 antigen diluted in 1.0 ml of complete RPMI; Mm206-89 received *env* variant 1-12 and Mm262-89 received *env* variant 8-22. The variant viruses used for inoculation had been grown in CEMx174 cells for a total of 12 days. The amount of p27<sup>gag</sup> protein was measured by the Coulter SIV Core Ag Assay (Coulter Corp., Hialeah, Fla.). Cell-free virus stocks for inoculation were prepared by centrifugation of infected cell cultures and filtration of viral supernatants through 0.45- $\mu$ m-pore-size filters. Heparinized blood samples were collected from the macaques at intervals after virus inoculation and were used for virus recovery and for monitoring antibody response. Infection of Mm243-86, Mm135-88, and Mm206-86 with SIVmac239 has been described previously (4, 20).

**Sera.** Sequential blood samples were collected at various intervals after virus inoculation. The whole blood was separated by centrifugation at 1,500 rpm for 10 min. Serum samples were heat inactivated at 56°C for 30 min, aliquoted, and stored frozen at either -20 or -70°C. The samples were quickly thawed at 37°C, and serial twofold dilutions were made in complete RPMI prior to neutralization.

**Construction of recombinant viruses.** The pBS subclones of SIVmac239 have been described previously (18). Briefly, SIVmac239 provirus was subcloned into 5' left-half (p239SpSp5') and 3' right-half (p239SpE3') plasmids through the use of a unique *Sph*I site located near the center of the viral genome (Fig. 1). The 5' clone used in the present study was obtained from the original stock (18). The 3' plasmid was modified to construct the recombinant viruses. The first modification consisted of removing two *Sst*I sites located in the flanking cellular sequences of the 3' plasmid (Fig. 1, triangle 1). This was achieved by cleaving the 3' plasmid with *Afl*II and *Bst*XI, which cut uniquely in the flanking cellular sequences outside the region containing the two *Sst*I sites. This restriction cleavage deleted 1.9 kb of the 3'-flanking cellular sequences. The 5' overhang left by *Afl*II was filled in with Klenow, and the 3' overhang left by *Bst*XI was removed with S1 nuclease. The blunt ends were ligated, and the ligation mixture was used to transform JM109 competent bacterial cells. A clone (provided by Hilary G. Morrison, New England Regional Primate Research Center) was selected on the basis of the absence of the two *Sst*I sites. A bacterial stock of this clone, called p239SpE3'( $\Delta$ SstI), was used to prepare plasmid DNA (Qiagen, Inc., Chatsworth, Calif.). The *Sph*I-*Eco*RI fragment of p239SpE3'( $\Delta$ SstI), containing all of the 3' viral sequences, was gel purified for later use. The second modification of the 3' plasmid consisted of removing a *Hind*III site located upstream of the *Sph*I site in the pBS polylinker region (Fig. 1, triangle 2). A two-step procedure was used for removing the *Hind*III site. In the first step, the pBS(-) vector (Stratagene, La Jolla, Calif.) was cleaved with *Hind*III, filled in with Klenow, ligated, and transformed into *Epicurian coli* XL1-Blue competent bacterial cells (Stratagene). A bacterial colony containing a pBS(-) vector with no *Hind*III site was selected. The pBS( $\Delta$ HindIII) vector was cleaved by *Sph*I and *Eco*RI and was ligated with the gel-purified *Sph*I-*Eco*RI fragment of

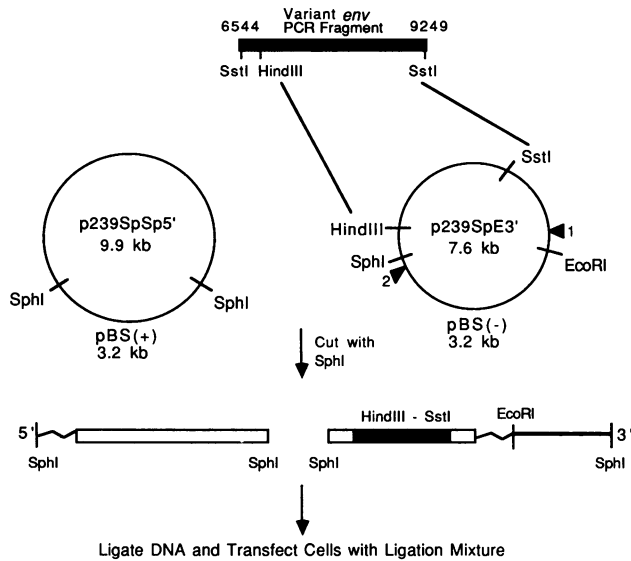


FIG. 1. Construction of recombinant viruses. The variant envelope gene is shown at the top as an *SstI-SstI* fragment. The numbering system is that of Regier and Desrosiers (45). The 5' and 3' subclones of parental SIVmac239 are shown as circles and have been described previously (18). The relative locations of important restriction enzyme sites are indicated. Numbered triangles indicate modifications of the parental plasmids as described in Materials and Methods. Fragments resulting from cleavage with the enzyme *SphI* are shown. Parental virus sequences are indicated by open boxes, and variant *env* sequences are shown as a solid box. The wavy line represents flanking cellular sequences, and the solid line represents pBS vector sequences. This figure is drawn approximately to scale.

p239SpE3'( $\Delta SstI$ ) (described above). The ligation mixture was used to transform *E. coli* XL1-Blue competent bacterial cells. A clone, called p239SpE3'( $\Delta HindIII\Delta SstI$ ), was selected. This plasmid served as the parental right-half clone and served as the backbone for construction of all variant viruses. To obtain the parental backbone, plasmid p239SpE3'( $\Delta HindIII\Delta SstI$ ) was cut with *HindIII* and *SstI*. The 2.4-kb *HindIII-SstI* parental envelope fragment was purified away from the 5.2-kb backbone by agarose gel electrophoresis. Variant envelope fragments were obtained by cutting the original polymerase chain reaction (PCR) clones with *HindIII* and *SstI*. The 2.4-kb *HindIII-SstI* variant *env* fragments were ligated with the 5.2-kb parental backbone, and the ligation mixture was used to transform *E. coli* XL1-Blue competent bacterial cells. The initial clones were selected on the basis of restriction enzyme analysis. The final recombinants selected were sequenced in the V1 and V4 regions of *env* to confirm each variant sequence.

**Transfection.** Since the complete viral genome was present on two plasmids, DNA segments were joined prior to transfection. A 3- $\mu$ g portion of each subclone was cut with *SphI* (Fig. 1). The enzyme was heat inactivated at 68°C for 10 min, and the DNA was precipitated with ethanol. The DNA pellet was resuspended in ligase buffer, and T4 DNA ligase was added. The ligation reaction was incubated overnight at 15°C. After ligation, the DNA was precipitated with ethanol and resuspended in 12  $\mu$ l of Tris-EDTA (TE) buffer. The DNA was added to 1.4 ml of DME-DEAE-dextran solution (Dulbecco's modified Eagle [DME] medium containing 125  $\mu$ g of DEAE-dextran per ml and 50 mM Tris [pH 7.3]). A 12- $\mu$ l solution of TE only (no DNA) was added to 1.4 ml of

DME-DEAE-dextran solution to serve as a negative control. The DME-DEAE-dextran solution containing either DNA or TE was used to resuspend  $5 \times 10^6$  CEMx174 cells which had been split 24 h prior to use. The cells were incubated with the DNA solution for 40 min at 37°C. After incubation, cells were washed once with serum-free DME medium and once with serum-free RPMI 1640 medium. The cells were resuspended finally in 10 ml of complete RPMI medium. The cultures were split twice weekly at ratios of 1:2 or 1:3. The cultures were monitored visually for cytopathic effect and were tested at various intervals after transfection for the presence of viral p27<sup>gag</sup> protein by using the Coulter SIV Core Ag Assay.

**Virus stocks for neutralization.** Virus stocks for use in neutralization were prepared from infected CEMx174 cells approximately 9 to 13 days postinfection. Before the virus was harvested, infected cells were pelleted and the supernatant was discarded. Complete RPMI medium was added, and the infected cells were resuspended at a density of  $6.0 \times 10^5$  viable cells per ml (half-maximal density for CEMx174). The virus was harvested 48 h later by clarifying the supernatant by centrifugation and filtering through 0.45- $\mu$ m-pore-size filters. Virus was stored in aliquots of 0.5 to 1.0 ml in a liquid nitrogen vapor tank at approximately -150°C.

**Titration of virus.** All virus stocks were titered on cloned MT4 cells to determine the amount of virus to be used in the neutralization assay. Titrations were performed in 96-well plates (Falcon MicroTest III) by using serial fivefold dilutions of virus in replicates of seven. The highest dilution of virus for which all wells were positive for cell killing was selected as the amount of virus to be used for neutralization. The dilutions selected for virus stocks of SIVmac239, T69BL1-12, and T93V8-22 were 1:100, 1:50, and 1:12.5, respectively.

**Cloning of MT4 cells.** MT4 cells were cloned by limiting dilution in 96-well plates by using a feeder layer of irradiated MRC-5 cells (ATCC 55-X; American Type Culture Collection). Approximately  $10^4$  irradiated MRC-5 cells were added to each well in 100  $\mu$ l of complete BME. This feeder layer of cells was incubated at 37°C in a CO<sub>2</sub> incubator. After 24 h, the medium was changed to complete RPMI (100  $\mu$ l). MT4 cells were diluted the next day and added to the feeder layer. The MT4 cells were counted several times, and the counts were averaged. Three different dilutions were made to yield a theoretical distribution of 0.167, 0.5, and 1.5 cells per well in a volume of 50  $\mu$ l. Then 50  $\mu$ l of each dilution was added to each of 48 wells. Since it was not possible to distinguish a single cell on the feeder layer, wells were screened for clusters of growing MT4 cells several days after plating. Every 2 to 5 days, a portion of the medium was changed. A mixture of fresh medium and filtered-conditioned medium was added to the plates (one-third conditioned medium from MT4 cells, one-third conditioned medium from MRC-5 cells, and one-third fresh RPMI 1640 medium). As the cell numbers increased, cells were transferred to larger tissue culture vessels and only fresh RPMI 1640 medium was added to the cultures. When the volumes became large enough and the passage number was still low, the cells were frozen by standard methods and stored in a liquid-nitrogen vapor tank at approximately -150°C.

**MTT neutralization assay.** Neutralization tests were performed by a serum dilution-constant virus method in 96-well plates. The assay used was modified from one described previously (7). Sera were heat inactivated prior to dilution. A 25- $\mu$ l portion of the appropriate virus dilution, containing 10 to 20 50% cell killing doses for MT4 cells, was mixed with 25

$\mu$ l of each serum dilution for the neutralization step. The virus-serum mixture was incubated in a CO<sub>2</sub> incubator. After 1 h at 37°C,  $3.0 \times 10^3$  cloned MT4 cells (50  $\mu$ l) were added and the plates were returned to the CO<sub>2</sub> incubator. On days 4, 7, and 10, fresh RPMI 1640 medium (50  $\mu$ l) was added to the plates. On day 14, the cells were assayed for viability by using a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Chemical Co.)] assay (6, 8, 33, 46). MTT is a yellow substrate which is cleaved by the mitochondria of living cells to yield a purple formazan product. For the MTT assay, 150  $\mu$ l of culture supernatant was carefully removed from each well of the neutralization plate and 30  $\mu$ l of MTT solution (1.67 mg/ml in phosphate-buffered saline) was added to each well. The plates were returned to the incubator for 4 h, during which time black MTT formazan crystals formed in the wells containing live cells. After the 4 h, 100  $\mu$ l of 0.04 N HCl in isopropanol was added to each well and vigorously mixed by repeated pipetting with a multichannel pipettor. HCl converted the phenol red in the medium to a yellow color that did not interfere with the MTT formazan measurement, whereas the isopropanol dissolved the formazan crystals to give a homogeneous purple color suitable for absorbance measurement. Within 1 h, the absorbance of the plates was read on a Dynatech MR5000 dual-wavelength enzyme-linked immunosorbent assay reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The neutralization titer was determined by taking the reciprocal of the highest dilution of serum that resulted in at least 60% maximal viability. The stated serum dilutions are those present in the initial neutralization step prior to the addition of cells.

**Radioimmunoprecipitation.** Radioimmunoprecipitation assays were performed as described by Kanki et al. (16). Briefly, CEMx174 cells infected with cloned viruses were metabolically labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. Virus was pelleted by high-speed centrifugation of cell-free supernatant. Virions were lysed with detergent, and the radiolabeled envelope glycoproteins were precipitated by using an SIV<sup>+</sup> rhesus monkey serum previously bound to protein A-Sepharose CL-4B (3, 17). The complex was washed, and the labeled protein was eluted in sample buffer by boiling at 100°C for 3.5 min. The samples were analyzed in 7.5% polyacrylamide gels. The gels were fixed, dried, and exposed to Kodak XAR film.

**DNA sequencing.** The double-stranded plasmid clones were sequenced by the primer-directed dideoxy-chain termination method (48) with Sequenase (United States Biochemical Corp., Cleveland, Ohio) and internal oligonucleotide primers synthesized on a Cyclone DNA Synthesizer (Biosearch, Inc., Burlington, Mass.) <sup>35</sup>S-labeled sequencing reactions were electrophoresed on 6% polyacrylamide gels with 8 M urea. Sequences were analyzed with IBI-Pustell DNA analysis software.

**Nucleotide sequence accession number.** The nucleotide sequences (data not shown) of the TM coding regions for variants T69BL1-12 and T93V8-22 have been filed with GenBank as updates to files under accession numbers M61078 and M61092, respectively.

## RESULTS

**Recombinant viruses.** Seven late-time-point envelope clones from Mm243-86 were selected for evaluation on the basis of unique sequence differences in five variable regions of gp120 (4). The *HindIII-SstI* fragments of the original clones were inserted into the parental backbone sequence as

described in Materials and Methods. The *HindIII-SstI* fragment encompasses nearly the entire envelope gene including both *SU* and *TM*. The *HindIII* enzyme cuts at nucleotide 6822 in *SU* (at amino acid 73, between Glu and Ser [Fig. 2]). The *HindIII* cut is upstream of the V1 region; therefore the *HindIII-SstI* variant *env* fragments contained all five variable regions of gp120. The *SstI* enzyme cuts at nucleotide 9230, three amino acids upstream of the stop codon of *TM* (Fig. 2); this was the original *SstI* site used for cloning the PCR-amplified material from the rhesus monkeys.

All seven recombinant clones were used to transfect CEMx174 cells, a CD4<sup>+</sup> human cell line sensitive to SIVmac infection. Transfected cultures were monitored visually for cytopathic effect, and portions of the culture supernatants were tested for viral p27<sup>gag</sup> protein. Three of the seven variant viruses (T69BL1-12, T93V8-22, and T93BL3-18) were found to be replication competent in this cell line. Relative to the other viruses, variant 3-18 appeared to replicate less efficiently in CEMx174 cells; the extent of cytopathic effect and the level of p27<sup>gag</sup> protein were less in cultures that received the 3-18 variant. Variants 1-12 and 8-22 were thus chosen for further neutralization studies. All of the remaining cultures, including cells transfected with TE buffer, were maintained for a total of 30 days, at which time they were still negative for p27<sup>gag</sup> protein. Recombinant clones which were negative for virus production in this cell line include T69BL1-21, T69LN2-32, T69V7-16, and T93BL3-25.

**Envelope sequences of molecularly cloned variants.** The envelope genes of variants 1-12 and 8-22 were sequenced completely, and the deduced amino acid sequences were compared with those of the parental clone, SIVmac239 (Fig. 2). Within the *HindIII-SstI* envelope fragment used for constructing the recombinants, there are 23 amino acid differences between SIVmac239 and variant 1-12 (18 in gp120 and 5 in gp41). There are 28 amino acid differences between SIVmac239 and variant 8-22 (18 in gp120 and 10 in gp41). This represents 96.6% amino acid identity in gp120 and 97.2 to 98.6% identity in gp41 for the variants relative to the parental clone. Variants 1-12 and 8-22 have 17 amino acid differences from each other in gp120 (96.8% identity) and 7 amino acid differences in gp41 (98.0% identity). The vast majority of amino acid substitutions and deletions in gp120 are clustered in five variable regions (Fig. 2). In gp41, amino acid substitutions cluster around the SF8/5E11 neutralization determinant (21) and between amino acids 792 and 804 of the cytoplasmic domain (Fig. 2).

**Infection of rhesus monkeys with variant viruses.** Recombinant viruses expressing the 1-12 and 8-22 variant envelope sequences were each inoculated into a juvenile rhesus monkey (Mm206-89 and Mm262-89, respectively), and both monkeys became infected. The monkeys were known to be infected since SIV was repeatedly isolated from their blood over time and they developed strong stable antibody responses to the virus (data not shown). Serum samples from these two monkeys were obtained regularly and stored for later use in neutralization tests. Mm206-89, inoculated with variant 1-12, was sacrificed when death appeared imminent after persistent infection with SIV for 1.3 years. Mm262-89, inoculated with variant 8-22, is currently alive at 1.8 years postinfection. At this time, CD4<sup>+</sup> cells in Mm262-89 represented 29% of peripheral blood mononuclear cells and the CD4/CD8 ratio was 0.7.

**Neutralization assay for cloned SIVmac.** There are no neutralization assays reported in the literature which describe the use of cloned SIV with serum from SIV-infected

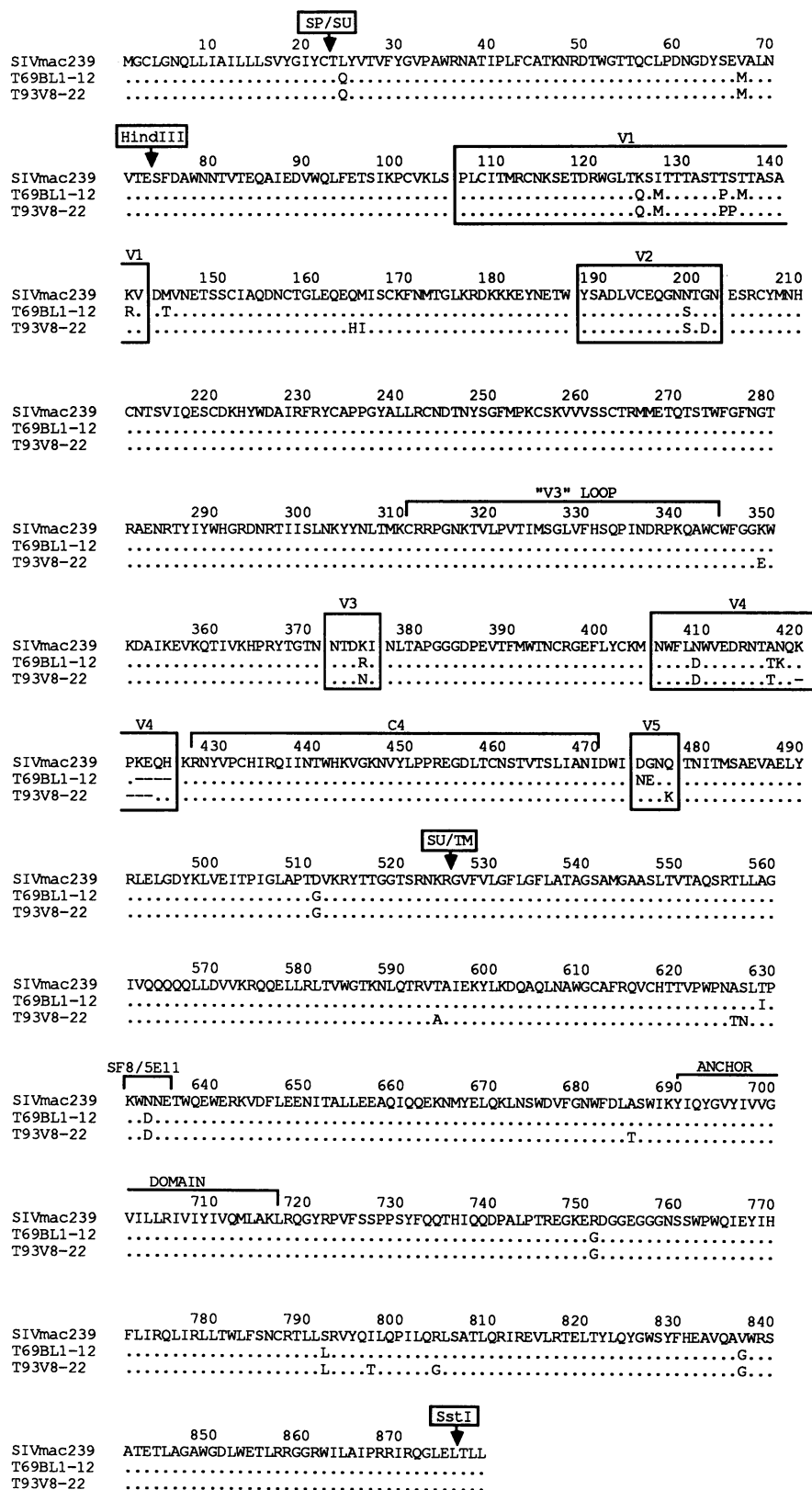


FIG. 2. Deduced amino acid sequences of the envelopes of SIVmac239, variant 1-12, and variant 8-22. Dots represent amino acid identity, and dashes represent deletions. Variable regions V1 through V5 are boxed (4). Brackets labeled "V3" LOOP and C4 refer to the V3 cysteine loop, which is variable in HIV-1 (49) and the fourth conserved region of HIV-1 which is important for CD4 binding (24). The region marked SF8/SE11 is a weak, strain-specific neutralization determinant of SIV (21). The signal peptide (SP) cleavage site (52) and the putative anchor domain (21) are those of SIV. SU, surface protein (gp120); TM, transmembrane protein (gp41) (26). The *Hind*III and *Sst*I sites used for cloning the variant envelopes into the parental virus are indicated.

TABLE 1. Reciprocal neutralization experiments

Serum sample and time p.i. <sup>a</sup> (wk)	Neutralization titer against:		
	SIVmac239	T69BL1-12	T93V8-22
239 antisera, Mm243-86			
0	<20	<20	<20
13	<20	<20	<20
23	<20	<20	<20
37	80	<20	<20
55	640	40	<20
79	160	40	<20
102	80	20	20
123	80	80	40
149	40	<20	<20
1-12 antisera, Mm206-89			
0	<20	<20	<20
18	<20	160	<20
38	20	160	20
46	40	80	20
55	<20	20	<20
8-22 antisera, Mm262-89			
0	<20	<20	<20
18	<20	20	160
38	40	20	160
46	20	20	160
55	<20	<20	20

<sup>a</sup> p.i., postinfection.

monkeys. In developing a neutralization assay for cloned SIV, one is limited not only by lack of appropriate simian cell lines but also by lack of permissive cell lines in general. Rhesus monkey peripheral blood lymphocytes support the growth of cloned SIVmac; however, only limited numbers of peripheral blood lymphocytes can be obtained from a rhesus monkey at one time and stable growth in culture occurs for only a limited time span. Cloned SIVmac does replicate well, however, in CEMx174 and MT4 cells.

A neutralization assay based on killing of MT4 cells by uncloned SIVmac251 virus has been described previously (7). Cloned SIVmac could not be used in this assay because the cloned virus did not kill enough MT4 cells to score in this assay. To obtain cells sufficiently sensitive to the killing effects of cloned virus, the MT4 cell line was cloned as described in Materials and Methods. One cloned cell line, called MT4 DB1-1, was used for further studies.

Virus titer determinations were performed with the cloned MT4 cells. With 300,000 cells/ml as described previously (7), complete cell killing was not achieved with the cloned MT4 cells. However, unlike the uncloned MT4 cells, cloned MT4 cells were capable of growing when seeded at low cell densities (as low as 30,000 cells per ml) with no effect on viability. When virus titer determinations were performed with cloned MT4 cells at the lower cell density of 30,000 cells per ml, complete cell killing was achieved for all three cloned viruses (parental SIVmac239, variant 1-12, and variant 8-22). Using the appropriate dilutions for each virus, pilot neutralization tests revealed that sera from SIV-infected monkeys could neutralize cloned SIVmac and protect cloned MT4 cells from cell death. This neutralization assay was therefore used for further experiments.

**Emergence of neutralization-resistant variants.** By using the MTT neutralization assay, neutralization titers were determined for sequential serum samples from five SIV-infected rhesus monkeys to determine whether neutralization-resistant variants had emerged during the course of

TABLE 2. Control neutralization experiments

Serum sample and time p.i. <sup>a</sup> (wk)	Neutralization titer against:		
	SIVmac239	T69BL1-12	T93V8-22
239 antisera, Mm135-88			
12	<20	<20	<20
20	80	<20	<20
52	640	<20	<20
111	20	<20	<20
131	80	<20	<20
239 antisera, Mm206-86			
12	<20	<20	<20
24	<20	<20	<20
53	320	<20	<20
85	80	<20	<20
137	20	<20	20

<sup>a</sup> p.i., postinfection.

persistent infection. All of the sera were tested against three cloned viruses (Tables 1 and 2). The same virus stock of each virus was used for all neutralization tests.

Sequential sera from Mm243-86, infected with parental cloned SIVmac239, were found to neutralize SIVmac239 much better than they neutralized variant viruses 1-12 and 8-22 (Table 1). The variant envelopes of 1-12 and 8-22 were cloned from Mm243-86 at 69 and 93 weeks postinfection, respectively. Sera taken either around or after the time of isolation of these variant clones had detectable levels of neutralizing antibodies against the variants, whereas sera taken prior to cloning did not yield measurable neutralization titers. Thus, there appeared to be delayed neutralizing-antibody responses against the variants in Mm243-86. The titers of neutralizing antibodies against variant viruses in the late-time-point sera from Mm243-86, however, never reached as high a level as against parental SIVmac239.

Sequential sera from two other animals infected with parental virus (Mm135-88 and Mm206-86) were also tested (Table 2). Sera from these two animals also neutralized the parental virus SIVmac239 much better than they neutralized the two variant viruses (Table 2).

Neutralization titers of sera from Mm243-86, Mm135-88, and Mm206-86, all infected with SIVmac239, peaked at approximately 1 year postinfection and subsequently declined as the animals progressed toward AIDS (Tables 1 and 2). The latest serum sample from Mm243-86 was taken at the time of death from AIDS (149 weeks). The latest samples from Mm135-88 and Mm206-86 (131 and 137 weeks, respectively) were taken just before death when the animals were showing signs of AIDS.

Since neutralization titers against any particular virus are not absolute measurements in any sense, the data with sera from Mm243-86, Mm135-88, and Mm206-86 in Tables 1 and 2 cannot by themselves be used to argue for the emergence of neutralization-resistant variants during persistent infection of Mm243-86. It is possible, for example, that stocks of the 1-12 and 8-22 variants are less efficient than SIVmac239 at scoring in this neutralization assay.

To rule out this possibility and to control internally for the results, reciprocal neutralization studies were performed with sera from animals infected with variant virus. Results from these cross-neutralization tests revealed that each variant was indeed neutralized best by sera from monkeys infected with the homologous virus (Table 1). Sera from Mm206-89, for example, which was inoculated with the 1-12 virus, neutralized 1-12 much better than they neutralized the

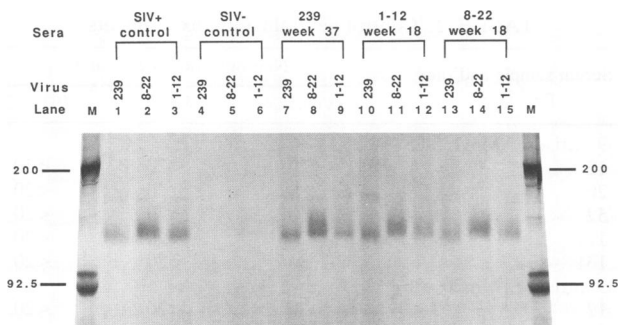


FIG. 3. Radioimmunoprecipitation. Virus was concentrated by centrifugation of supernatants from radiolabeled cells infected with one of three cloned viruses: parental SIVmac239, variant 1-12, and variant 8-22. Virions were disrupted with detergent and were immunoprecipitated with the following polyclonal sera: SIV(+) control sera from an SIV-positive monkey; SIV(-) control sera from an SIV-negative monkey; SIVmac239 week 37, sera from animal Mm243-86 infected with cloned SIVmac239; 1-12 week 18, sera from animal Mm206-89 infected with cloned variant 1-12; and 8-22 week 18, sera from animal Mm262-89 infected with cloned variant 8-22. Lanes labeled M represent molecular mass markers expressed in kilodaltons.

other cloned viruses (Table 1). The antisera from Mm206-89 neutralized SIVmac239 and 8-22, but neutralization was delayed and the titers were lower. Similarly, sera from Mm262-89, which was inoculated with the 8-22 virus, neutralized 8-22 better than they neutralized the other cloned viruses. The antisera from Mm262-89 also neutralized SIVmac239 and 1-12, but again neutralization was delayed and the titers were lower against these viruses. At certain time points postinfection (week 18, for example), neutralization titers of the 1-12 and 8-22 sera were as much as eightfold higher (or more) against homologous virus than against heterologous virus (Table 1).

Neutralization titers of sera from the two animals that received variant viruses peaked slightly earlier than those of sera from the three animals that received parental virus; neutralization titers reached their highest levels by 18 to 46 weeks postinfection and had declined to lower levels after 1 year postinfection (Table 1). Mm206-89 died at 66 weeks postinfection, whereas Mm262-89 is alive at 92 weeks postinfection.

**Radioimmunoprecipitation.** Week 37 serum from Mm243-86 and week 18 sera from Mm206-89 and Mm262-89 displayed dramatic differences in neutralization of the homologous virus versus the heterologous virus (Table 1). The neutralization titer of week 37 sera against the parental virus was 80, while it was less than 20 against each of the two variant viruses. Conversely, the neutralization titer of week 18 sera against each variant virus was 160, while it was <20 against the parental virus. A radioimmunoprecipitation assay was performed to determine whether these dramatic differences in neutralization reflected differences in the ability of these sera to bind parental versus variant envelope sequences. The radioimmunoprecipitation assay revealed no significant difference in the ability of each sera to precipitate each of the three envelope proteins (Fig. 3).

## DISCUSSION

Seven recombinant clones expressing variant envelope sequences of SIVmac239 were constructed so that immune

selection during persistent infection could be studied. The seven envelopes, originally isolated from Mm243-86, were selected on the basis of unique sequence differences in five variable regions in gp120 (4). Three of seven recombinants were replication competent in the CD4<sup>+</sup> human cell line CEMx174. Although there were no obvious defects in the envelope sequences of the four viruses which did not replicate (such as stop codons or frameshifts), it is possible that some of the individual amino acid changes resulted in the inability of virus to replicate. Since the recombinants have been tested on only one human cell line, it is also possible that some of the variants are replication competent but limited in their host cell range.

Because variant 3-18 appeared to replicate less efficiently than the other viruses, our immune selection studies focused on variants 1-12 and 8-22. As its full name implies, T69BL1-12 was cloned from PCR-amplified total-cell DNA prepared from peripheral blood mononuclear cells isolated at 69 weeks postinfection. Variant T93V8-22 was cloned from PCR-amplified Hirt supernatant DNA prepared from cells infected with virus isolated at 93 weeks postinfection. Using variants 1-12 and 8-22 along with SIVmac239, a neutralization assay was developed for these cloned viruses. The assay was adapted from a procedure described previously for uncloned SIVmac251 (7). Modifications of the assay for cloned SIVmac included the use of cloned MT4 cells and lower cell densities.

To analyze whether neutralizing-antibody responses are responsible at least in part for selecting amino acid changes in variable regions of env, it was necessary to determine neutralization titers for a variety of virus-serum combinations (Tables 1 and 2). One problem with comparing neutralization titers of a particular serum against three different viruses is normalization of the amount of virus. For the MTT neutralization assay, the amount of virus to be used was determined by finding the titer of the virus and taking the highest dilution of virus which repeatedly and completely induced cell killing. Therefore, the absolute amount of virus in each assay may not be the same. Differences in a variety of other parameters such as replication rate, spike density, CD4 affinity, and gp120-gp41 affinity may also affect the neutralization process and thus influence the neutralization titers that can be obtained with different viruses. For these reasons, one-way neutralization studies, such as those with the HIV-1 system (1, 13, 31, 51, 53), are not sufficient to prove that specific neutralization-resistant variants have emerged in individuals during persistent infection.

For the SIVmac studies described in this report, two-way "reciprocal" neutralization studies were performed. First, sera taken at various time points postinfection from three animals infected with parental SIVmac239 were tested against the three cloned viruses (Tables 1 and 2). Sera from all three animals neutralized SIVmac239 better than they neutralized either of the two variants. Sera taken from Mm243-86 at time points around or after the time of cloning of 1-12 and 8-22 neutralized each of the two variants, but the neutralization titers against the variants were lower than against the parental virus. For the reciprocal experiment, sera from animals infected with either 1-12 or 8-22 were used to show that each variant was neutralized best by its homologous antisera (Table 1). The resistance of the variants to neutralization is particularly impressive when one considers that they still share 96.6% amino acid identity in gp120 and 97.2 to 98.6% amino acid identity in gp41 with the parental SIVmac239. Radioimmunoprecipitation analysis (Fig. 3) demonstrated that the dramatic differences in neu-

tralization of the viruses were not associated with any difference in the overall anti-gp120 antibody reactivity. Therefore, if gp120 is indeed the major target of neutralizing-antibody responses, neutralizing antibodies probably represent only a small fraction of the total anti-gp120 antibody population. The SIVmac immune-selection studies presented here offer the most definitive evidence to date that neutralization-resistant variants emerge in an individual during persistent infection with a primate lentivirus.

Reciprocal neutralization tests were reported in one previous EIAV study. Kono et al. (23) had previously inoculated each of five horses with five different variant viruses; the variant strains had been isolated over time from one chronically infected horse. There were delayed neutralizing-antibody responses against variant strains of EIAV in the original animal, and type-specific neutralizing-antibody responses were generated in each of the five horses inoculated with variant viruses. Although some of the sera displayed a significant amount of cross-reactivity, each EIAV variant was neutralized best by its homologous antisera. Results with molecularly cloned SIVmac are similar to these early results with uncloned EIAV. The availability of cloned reagents in the SIV system will now allow us to define genetic determinants for neutralization escape and to further evaluate the selective forces operating in vivo during persistent infection. Results from this defined system should allow a clearer understanding of the role of antigenic variation and immune selection in viral persistence and the pathogenesis of AIDS.

Attempts to identify neutralization epitopes in the SIV system indicate that conformational determinants may be more important than linear determinants (15). Although the V3 loop is often referred to as the principal neutralizing determinant of HIV-1, there is no clear evidence to suggest that V3 epitopes predominate over conformational epitopes as targets of neutralizing antibody responses during the natural course of HIV-1 infection in humans. It is not yet known to what extent neutralizing antibodies in patient sera are directed at linear versus conformational epitopes, variable versus conserved, or V3 versus other domains. Haigwood et al. (12) have presented evidence for the role of at least three HIV-1 gp120 variable regions in recognition by neutralizing antibodies. The cloned SIV variants and other reagents described in this report will allow detailed investigation of these issues at least in the SIV system.

The studies with SIVmac presented in this paper indicate that the humoral branch of the immune system is at least one of the selective forces operating on an array of variants present in vivo. Cytotoxic T-lymphocyte and antibody-dependent cell-mediated cytotoxicity responses could also conceivably select for variant viral envelope sequences (29, 44). In addition to immune selection, other selective forces may operate in vivo. For example, there may be selection for things such as host, cell, and tissue tropism, replication rate, syncytium-forming ability, CD4 affinity, gp120-gp41 affinity, and cytopathic effect. In light of the complexity of lentiviral diseases, there may indeed be multiple selective forces operating simultaneously on any one particular envelope region, and some of these selective forces may be stronger than others. The well-defined SIVmac system described in this report should facilitate precise delineation of which selective forces are most important for driving change in each variable region.

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