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Abstract: Neonatal rhesus macaque 95-3 was inoculated with nonpassaged simian-human immunodeficiency virus strain SHIV-vpu(+), which encodes env of the laboratory-adapted human immunodeficiency virus (HIV) strain IIIB and is considered nonpathogenic. CD4(+) T-cell counts dropped to <200 cells/microl within 4.6 years, and monkey 95-3 died with opportunistic infections 5.9 years postinoculation. Transfer of blood from 95-3 to two naive adult macaques resulted in high peak viral loads and rapid, persistent T-cell depletion. Progeny virus evolved in 95-3 despite high SHIV-vpu(+) neutralizing antibody titers and still used CXCR4 but, in contrast to parental SHIV-vpu(+), productively infected macrophages and resisted neutralization. Sequence analysis revealed three new potential glycosylation sites in gp120; another two were lost. Strikingly similar mutations were detected in a laboratory worker who progressed to AIDS after accidental HIV-IIIB infection (T. Beaumont et al., J. Virol. 75:2246-2252, 2001), thus supporting the SHIV-vpu(+)/rhesus macaque system as a relevant model. Similar mutations were also described after rapid passage of chimeric viruses encoding IIIB env in rhesus and pig-tailed macaques (M. Cayabyab et al., J. Virol. 73:976-984, 1999; Z. Q. Liu et al., Virology 260:295-307, 1999; S. V. Narayan et al., Virology 256:54-63, 1999; R. Raghavan et al., Brain Pathol. 7:851-861, 1997; E. B. Stephens et al., Virology 231:313-321, 1997). Thus, HIV-IIIB env evolved similarly in three different species; this selection occurred in chronically infected individuals during disease progression as well as after rapid virus passage. We postulate that evolutionary pressure led to the outgrowth of more aggressive viral variants in all three species.

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Molecular Evolution of Human Immunodeficiency Virus *env* in Humans and Monkeys: Similar Patterns Occur during Natural Disease Progression or Rapid Virus Passage

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Neonatal rhesus macaque 95-3 was inoculated with nonpassaged simian-human immunodeficiency virus strain SHIV-vpu$^+$, which encodes *env* of the laboratory-adapted human immunodeficiency virus (HIV) strain IIIB and is considered nonpathogenic. CD4$^+$ T-cell counts dropped to <200 cells/μl within 4.6 years, and monkey 95-3 died with opportunistic infections 5.9 years postinoculation. Transfer of blood from 95-3 to two naive adult macaques resulted in high peak viral loads and rapid, persistent T-cell depletion. Progeny virus evolved in 95-3 despite high SHIV-vpu$^+$ neutralizing antibody titers and still used CXCR4 but, in contrast to parental SHIV-vpu$^+$, productively infected macrophages and resisted neutralization. Sequence analysis revealed three new potential glycosylation sites in gp120; another two were lost. Strikingly similar mutations were detected in a laboratory worker who progressed to AIDS after accidental HIV-IIIB infection (T. Beaumont et al., J. Virol. 75:2246–2252, 2001), thus supporting the SHIV-vpu$^+$/rhesus macaque system as a relevant model. Similar mutations were also described after rapid passage of chimeric viruses encoding IIIB *env* in rhesus and pig-tailed macaques (M. Cayabyab et al., J. Virol. 73:976–984, 1999; Z. Q. Liu et al., Virology 260:295–307, 1999; S. V. Narayan et al., Virology 256:54–63, 1999; R. Raghavan et al., Brain Pathol. 7:851–861, 1997; E. B. Stephens et al., Virology 231:313–321, 1997). Thus, HIV-IIIB *env* evolved similarly in three different species; this selection occurred in chronically infected individuals during disease progression as well as after rapid virus passage. We postulate that evolutionary pressure led to the outgrowth of more aggressive viral variants in all three species.

Simian-human immunodeficiency viruses (SHIVs) contain envelope and accessory genes of HIV type 1 (HIV-1) in a SIV backbone (11, 16, 17, 26, 32, 33, 40). Several chimeric viruses with different pathogenic potentials have been constructed. Whereas some strains are highly pathogenic in rhesus macaques (17, 24, 33, 34), others are thought to be nonpathogenic, such as SHIV-4 and SHIV-vpu$^+$ (20, 21). The latter two chimeras encode *env* of HXBc2, a molecular clone of the T-cell line-adapted HIV-IIIB (20, 21). In contrast to SHIV-4, SHIV-vpu$^+$ contains an open *vpu* reading frame (20, 21). Both viruses replicate in rhesus monkeys (2, 25, 36).

Rapid in vivo passage of viruses containing HIV-IIIB *env* resulted in more aggressive variants that caused acute CD4$^+$ T-cell loss (15, 16, 31, 43). However, thus far, macaques inoculated with the nonpassaged viruses have not developed signs of immune suppression or disease (20, 21). Here, we demonstrate that nonpassaged SHIV-vpu$^+$ can cause CD4$^+$ T-cell depletion and AIDS after prolonged observation, thus approximating the time course of untreated HIV-1 infections in humans.

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Nonpassaged SHIV-vpu$^+$ induces AIDS. After nontraumatic oral SHIV-vpu$^+$ inoculation (<10 oral 50% animal infectious doses) (1), a neonatal rhesus monkey (*Macaca mulatta* 95-3) became systemically infected. Virus isolation from peripheral blood mononuclear cells (PBMC) (1, 22) was persistently positive from weeks 2 to 49 postexposure and from week 228 onward. Viral RNA load in plasma measured by real-time reverse transcription-PCR (RT-PCR) (13) continuously increased from week 150 postexposure onward to reach $>10^5$ copies/ml (Fig. 1A). After seroconversion, anti-Gag antibodies decreased again (Fig. 1B), a finding that heralds development of immunodeficiency in SIV-infected monkeys and HIV-infected humans (5). Interestingly, a transient rebound of the anti-Gag antibodies was found at 123 weeks postexposure (Fig. 1B). CD4$^+$ T cells steadily declined after week 150 (Fig. 1C) and were <50 cells/μl during the last 7 months. The monkey developed substantial weight loss and diarrhea and was sacrificed at week 307. High viral burdens were detected by cocultivation in lymphoid tissues (data not shown). Gross necropsy and histology demonstrated lymphadenopathy, pneumocystosis, and colitis with cryptitis. Infectious organisms found in this animal are listed in Table 1.

Neutralizing activity against parental SHIV-vpu$^+$ was initially low to moderate (Fig. 1D); the titers rose after viral RNA loads had increased and before CD4$^+$ T-cell counts fell below

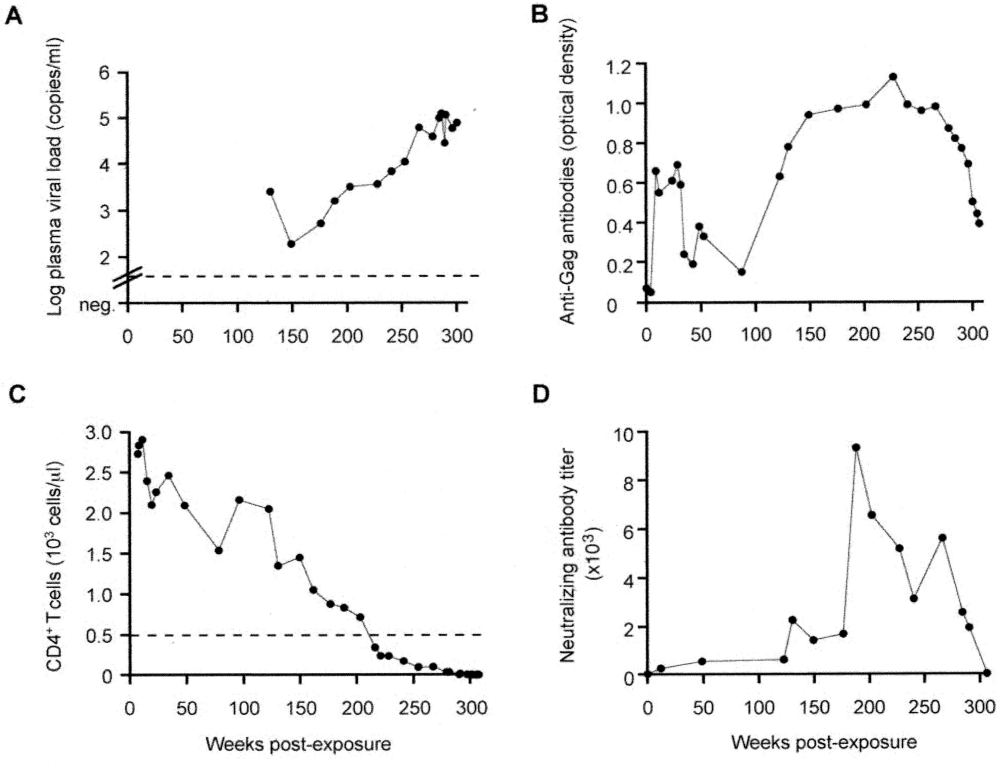

FIG. 1. SHIV-vpu$^+$ RNA load in plasma determined by real-time RT-PCR (A), anti-Gag antibodies (B), peripheral absolute CD4$^+$ T-cell counts (C), and neutralization of SHIV-vpu$^+$ in plasma from animal 95-3 (D). The sensitivity of the RT-PCR assay (A) is indicated by the dotted line (50 copies/ml). During the first 130 weeks postexposure, no plasma samples were available with an anticoagulant suitable for RT-PCR. In panel C, 500 CD4$^+$ T cells/μl are indicated by a dotted line. Anti-Gag antibodies were assessed by enzyme-linked immunodominant assay as described previously (1, 2, 22) and given as optical density. Homologous neutralization of plasma was tested in triplicate in a modified MT-2 cell assay with SHIV-vpu$^+$ (D) as described previously (19). Neutralizing antibody titers are the reciprocal dilution which protected 50% of the MT-2 cells from virus-induced cytotoxicity and correspond to 90% reduction of viral Gag synthesis (6). The lower limit of detection was determined as that giving a titer of 10.

500 cells/μl. Thus, progeny virus evolved in monkey 95-3 despite high SHIV-vpu$^+$ neutralizing antibody titers.

Parental SHIV-vpu$^+$ evolved into a neutralization-resistant and acutely pathogenic virus in animal 95-3. Virus isolated from animal 95-3 at necropsy was highly resistant to neutralization by plasma collected at weeks 180, 200, 225, 270, and 285 postinoculation (titers of <20), despite the presence of high titers of antibodies that neutralized parental SHIV-vpu$^+$ at all time points (data not shown). Animal passage revealed that monkey 95-3 harbored virus with markedly increased virulence after it had progressed to AIDS. Blood (10 ml containing 642 infectious PBMC and 7.4×10^5 RNA copies) was collected at week 291 postinoculation from monkey 95-3 and inoculated intravenously into adult recipient monkey RJj-4. The animal developed high virus loads, rapid CD4$^+$ T-cell losses in blood (Fig. 2) and lymphoid tissue (data not shown), and refractory diarrhea (Table 1). At week 43, RJj-4 had 24 CD4$^+$ T cells/μl and was euthanatized because of *Pneumocystis carinii* pneumonia. A second blood transfer (10 ml containing 1.3×10^5 infectious PBMC and 1.26×10^8 RNA copies) from RJj-4 at week 2 postexposure to animal RMk-4 also resulted in high viral loads and rapid CD4$^+$ T-cell depletion (Fig. 2). RMk-4 was sacrificed at week 58 due to *P. carinii* pneumonia.

TABLE 1. Outcome of SHIV-vpu$^+$ or progeny virus infection: infectious organisms

Monkey	Viral inoculum	Time of sacrifice (weeks after virus exposure)	Infectious agents
Neonate 95-3	SHIV-vpu$^+$	307	*Campylobacter coli* *Campylobacter jejuni* *Pneumocystis carinii*
Adult RJj-4	Blood from 95-3 collected at week 291	43	*Giardia lamblia* *Campylobacter coli* Adenovirus *Pneumocystis carinii*
Adult RMk-4	Blood from RJj-4 collected at week 2	58	*Pneumocystis carinii*

Unchanged CXCR4 usage but newly acquired ability to replicate in macrophages. Acute pathogenicity was also observed in pig-tailed macaques (*Macaca nemestrina*) after rapid passage of SHIVs encoding HIV-IIIB *env* (from HXBc2) (16, 43). A molecular clone, $SHIV_{KU\text{-}2MC4}$, isolated after further passage, caused acute CD4$^+$ T-cell loss and disease (23). Unlike the parental SHIV-4, which is not macrophagetropic, these progeny viruses replicated efficiently in macrophages (23, 44), even though they continued to use CXCR4 as coreceptor (23). We characterized coreceptor usage of viruses isolated from

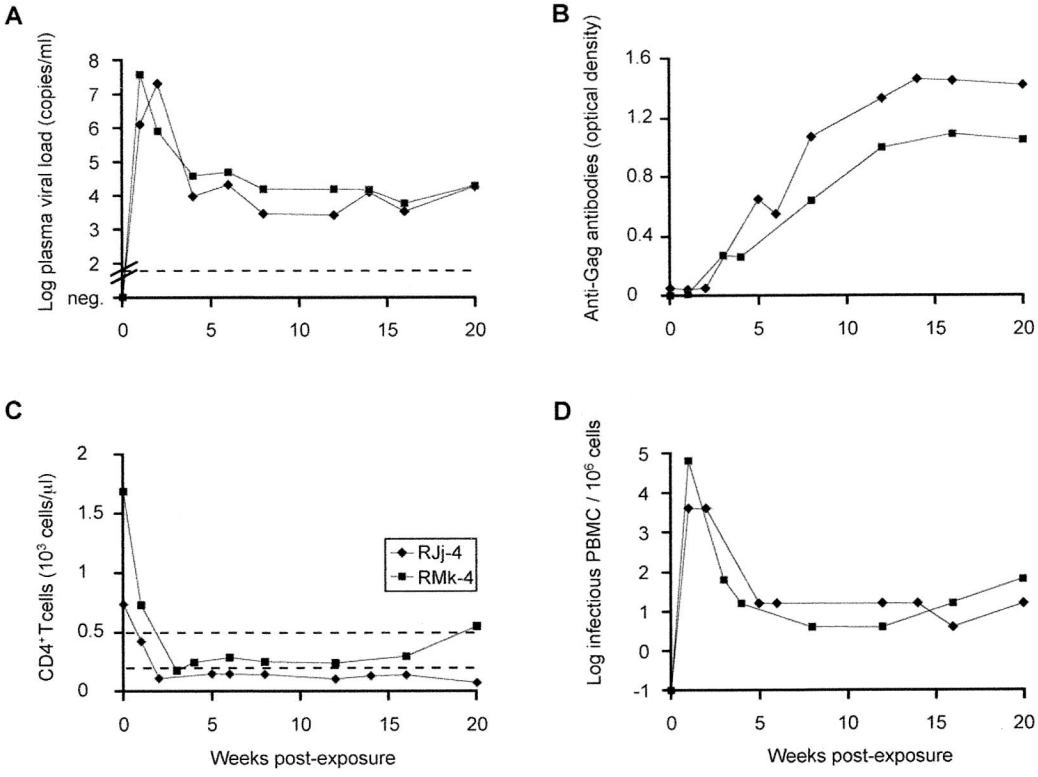

FIG. 2. Plasma SHIV-vpu$^+$ RNA load determined by real-time RT-PCR (A), anti-Gag antibodies assessed by enzyme-linked immunodominant assay (B), peripheral absolute CD4$^+$ T-cell counts (C), and virus isolation (D) for recipients RJj-4 and RMk-4. The sensitivity of the RT-PCR assay (A) is indicated by a dotted line (50 copies/ml). In panel C, 500 and 200 CD4$^+$ T cells/μl, respectively, are indicated by dotted lines.

95-3 at week 285 (SHIV-vpu$^+$5.285) and at necropsy (SHIV-vpu$^+$5.307) in U87 cells stably expressing various coreceptors (NIH AIDS Research and Reference Reagent Program). The progeny viruses productively infected CXCR4- but not CCR5-expressing cells. However, virus isolated at necropsy also acquired the capacity to replicate in rhesus macaque macrophages (data not shown). CXCR4 usage and replication in macrophages were also found in virus variants isolated from a laboratory worker who developed AIDS 8 years after accidental infection with HIV-IIIB (4). Thus, all of these virus variants had acquired the ability to productively infect macrophages despite unchanged coreceptor usage.

Sequence analysis of progeny viruses isolated from animal 95-3. Because earlier work had shown that replacing an *env* fragment from the acutely pathogenic SHIV$_{KU-1}$ was sufficient to convert the nonpathogenic parental SHIV-HXBc2 into a virus that caused rapid, profound CD4$^+$ T-cell loss (termed SHIV-HXBc2P3.2) (8), we decided to sequence a large gp120-encoding *env* segment. We used PCR to clone a 1,226-bp *env* fragment from PBMC DNA of monkey 95-3 collected at different time points. Two clones from week 2 (SHIV-vpu$^+$5.2) and eight clones from week 285 were sequenced using the following primers: GTAAAACGACGGCCAG, GT TCAATGGAACAGGACCAT, TTGGAGTACTGAAGGG TCAA (sense) and CAGGAAACAGCTATGAC, GTGT CACTTCCTTCAGTGT, ACATTGTACTGTGCTGACAT (antisense).

We found a higher number of amino acid substitutions and deletions in SHIV-vpu$^+$5.285 *env* (Fig. 3) than previously reported for rapidly passaged SHIV encoding HXBc2 *env* or viruses isolated from the HIV-IIIB-infected laboratory worker (4, 8, 23, 28, 44, 45). From 10 amino acid substitutions in gp120 linked to the conversion of parental SHIV-HXBc2 into the acutely pathogenic SHIV-HXBc2P3.2 (8), 5 were identical in SHIV-vpu$^+$5.285 (Table 2, highlighted in bold). Changes were mainly located in the variable regions of gp120 (Fig. 3). Many mutations were located in V3, a region that contains linear and discontinuous antigenic determinants (9, 18, 39) that can change during disease development and immune escape (37, 38, 41). Some amino acid substitutions led to three new and loss of two potential N-linked glycosylation sites, which in turn might result in changes in conformation and immune recognition of gp120 (3, 12, 14, 29, 30, 35, 47, 50). Two new glycosylation sites were located in V1 and V2 domains that contain epitopes for neutralizing antibodies (10, 27, 46) and thus can modulate neutralization sensitivity (7, 41, 48, 49). There were two FNTSW sequences in V4 of parental SHIV-vpu$^+$; one was lost by a 5-amino-acid deletion.

HIV-IIIB and SHIV encoding IIIB *env* undergo similar gp120 changes during chronic infection in humans or monkeys or during rapid in vivo passage. Some amino acid changes leading to alterations in potential glycosylation sites were reported also in viruses isolated after rapid passage or chronic infection from monkeys or humans (Table 2). Six amino acid changes found in gp120 of SHIV-vpu$^+$5.285 were identical to those found in gp120 of FF3346 (Table 2), a virus isolated from the HIV-IIIB-infected laboratory worker (4). These substitutions included a mutation in the highly antigenic sequence,

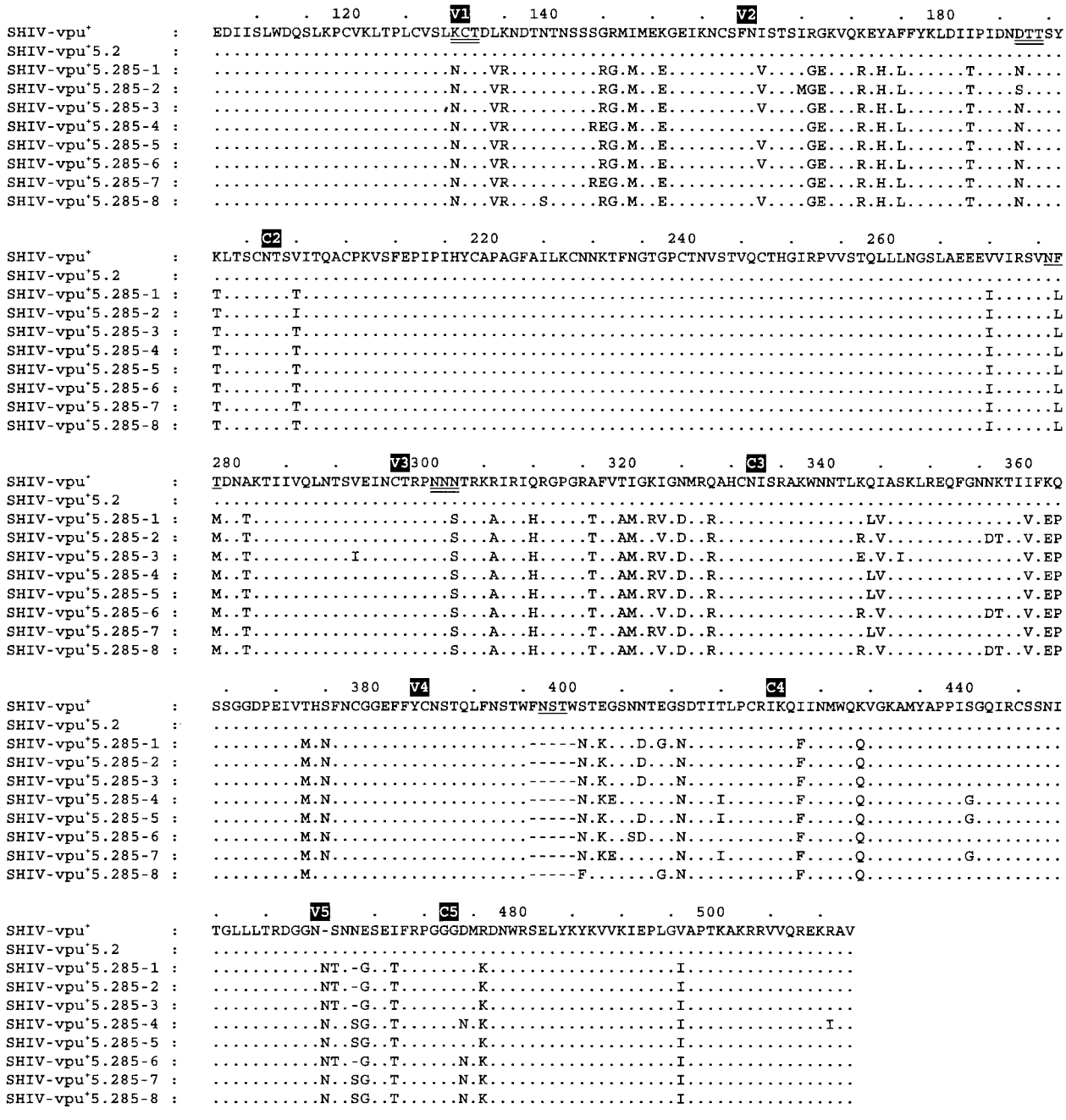

FIG. 3. Comparison of the gp120 sequence of the parental virus, SHIV-vpu$^+$, and the predicted gp120 amino acid sequences of SHIV-vpu$^+$5.2 isolated from monkey 95-3 at week 2 directly from PBMC and of SHIV-vpu$^+$5.285-1 to SHIV-vpu$^+$5.285-8 isolated from 95-3 285 weeks postexposure. Direct sequencing was not efficient from PBMC collected at week 285; thus, SHIV-vpu$^+$5.285 was amplified beforehand for 6 days in CEMx174/GFP cells. Differences in amino acid residues are indicated, as are the locations of the different regions of gp120 (4). The predicted gp120 amino acid sequence of two clones isolated at week 2 (SHIV-vpu$^+$5.2) was identical to that of the parental virus. The eight clones from week 285 had 39 consistent changes in gp120 when compared with the sequence of the parental virus: 6 in V1, 7 in V2, 9 in V3, 1 in V4, 3 in V5, and the other 13 in various constant regions. New potential N-linked glycosylation sites are double underlined; loss of potential N-linked glycosylation sites are single underlined. The gp120 sequence of SHIV-vpu$^+$ is identical to that of SHIV-4; the GenBank accession number of the latter is AF038399 (20).

GPGRAF, at the tip of the V3 loop (316A→T). An additional five changes were located identically in FF3346 and SHIV-vpu$^+$5.285, but the replacing amino acids were different (details in Table 2).

Summary. We described a general tendency of primate lentiviruses to mutate into more virulent forms over time by using an evolutionary path that was similar within individual hosts of different species. The virus that evolved in monkey 95-3 over several years of infection also resembled, to a certain degree, the viruses selected by rapid serial in vivo passage in pig-tailed and rhesus macaques. These latter viruses also induced acute CD4$^+$ T-cell depletion and AIDS in infected animals. In all

TABLE 2. Similar molecular evolution of HIV-IIIB *env* sequences in *M. mulatta, M. nemestrina, and H. sapiens*

SHIV-vpu$^+$5.285 amino acid changes[a] (*M. mulatta*)	Region	Site	SHIVs containing identical changes		HIV-IIIB variant with identical and/or similar changes (*H. sapiens*)
			M. mulatta	*M. nemestrina*	
130 K→N	V1	New PGS[b]	SHIV-HXBc2P3.2[c] (8), $SHIV_{KU-2MC4}$[c] (23), $SHIV_{KU-2}$[d] (23, 31)	$SHIV_{KU-1b}$[e] (28), $SHIV_{KU-PW1}$, $SHIV_{KU-8124}$, $SHIV_{KU-PEy}$[f] (42), $SHIV_{KU-1}$ Pnb[f] (44), $SHIV_{KU-1/105w52}$, $SHIV_{KU-1/105w98}$[g] (45)	
145 G→R	V1			$SHIV_{KU-1}$ Pnb1	
146 R→G	V1			$SHIV_{KU-1b}$, $SHIV_{KU-8124}$, $SHIV_{KU-PW1}$, $SHIV_{KU-1/105w98}$	FF3346[h] (4)
148 I→M	V1			$SHIV_{KU-1/105w52}$	
151 K→E	V1			$SHIV_{KU-8124}$, $SHIV_{KU-1/105w98}$	
161 I→V	V2			$SHIV_{KU-1/105w52}$	
166 R→G	V2				FF3346 (R→K)[h]
171 K→R	V2				FF3346 (K→E)
175 F→L	V2			SHIVKU-1b, $SHIV_{KU-PW1}$, $SHIV_{KU-PEy}$	
187 D→N	V2	New PGS			
192 K→T	V2		SHIV-HXBc2P3.2, $SHIV_{KU-2MC4}$	$SHIV_{KU-1b}$, $SHIV_{KU-PW1}$, $SHIV_{KU-8124}$, $SHIV_{KU-PEy}$, $SHIV_{KU-1}$ Pnb, $SHIV_{KU-1/105w52}$, $SHIV_{KU-1/105w98}$	FF3346
270 V→I	C2			$SHIV_{KU-1}$ Pnb5	
278 T→M	C2	Loss of PGS	SHIV-HXBc2P3.2, $SHIV_{KU-2MC4}$, $SHIV_{KU-2}$	$SHIV_{KU-1b}$, $SHIV_{KU-8124}$, $SHIV_{KU-1/105w52}$, $SHIV_{KU-1/105w98}$, $SHIV_{KU-1}$ Pnb3+10	
281 A→T	C2	CD4 binding		$SHIV_{KU-PW1}$	FF3346 (A→V)
302 N→S	V3	New PGS			
310 Q→H	V3			$SHIV_{KU-1b}$	FF3346
316 A→T	V3	GPGRAF			FF3346
320 I→M	V3		SHIV-HXBc2P3.2, $SHIV_{KU-2MC4}$, $SHIV_{KU-2}$	$SHIV_{KU-1b}$, $SHIV_{KU-PW1}$, $SHIV_{KU-8124}$, $SHIV_{KU-PEy}$, $SHIV_{KU-1}$ Pnb, $SHIV_{KU-1/105w52}$, $SHIV_{KU-1/105w98}$	
325 N→D	V3		SHIV-HXBc2P3.2, $SHIV_{KU-2}$	$SHIV_{KU-PW1}$, $SHIV_{KU-8124}$, $SHIV_{KU-PEy}$, $SHIV_{KU-1}$ Pnb, $SHIV_{KU-1/105w52}$, $SHIV_{KU-1/105w98}$	
328 Q→R	V3			$SHIV_{KU-1/105w52}$, $SHIV_{KU-1/105w98}$	
345 I→V	C3				FF3346
362 K→E	C3				FF3346 (K→R)
396–400 ΔFNSTW	V4	Loss of PGS		$SHIV_{KU-PW1}$, $SHIV_{KU-1}$ Pnb3+10	
429 K→Q	C4	CD4 binding			FF3346 (K→E)
464 E→G	V5				FF3346
467 I→T	V5			$SHIV_{KU-PEy}$	
474 D→N	V5			$SHIV_{KU-PEy}$, $SHIV_{KU-PW1}$	
476 R→K	V5			$SHIV_{KU-PEy}$, $SHIV_{KU-PW1}$	
496 V→I	C5			$SHIV_{KU-1b}$	

[a] Comparison with SHIV-4, AF038399 (20). Changes are listed that concerned potential glycosylation sites or had been reported in other viruses also. Bold type indicates 5 of 10 amino acid substitutions in SHIV-HXBc2P3.2 gp120 that were reported to be sufficient to confer disease (8).
[b] PGS, potential glycosylation site.
[c] Molecular clones acutely pathogenic in rhesus macaques (8, 23).
[d] Virus stock pathogenic in rhesus macaques (23, 31).
[e] Neutralization escape variant isolated from a pig-tailed macaque (28).
[f] Viruses isolated from pig-tailed macaques (42, 44).
[g] Immune escape virus isolated from a macaque (45).
[h] Virus isolated from a laboratory worker 7 years after accidental HIV-1 IIIB infection (4). For FF3346, changes are given that are located identically but with different replacing amino acids.

three species (*M. mulatta, M. nemestrina*, and *Homo sapiens*), progeny viruses became more neutralization resistant (4, 8, 28, 41) and acquired the ability to replicate in macrophages while maintaining CXCR4-restricted coreceptor usage (4, 23, 44). Thus, infection with nonpassaged SHIV-vpu$^+$ in an individual macaque (i) seems to approximate the evolution of HIV-IIIB seen during the course of disease progression in a human individual and (ii) is mirrored by the more rapid evolution that the virus undergoes during rapid serial passages in nonhuman primates.

Our results support the use of SHIV-vpu$^+$ infection of rhesus monkeys as a relevant model that can yield important insights into viral evolution and pathogenesis. Furthermore, since rapid viral passage is less time-consuming than long-term follow-up over years, it seems justifiable to use the former for safety testing of candidate live attenuated virus vaccines to uncover potential virulence.

Nucleotide sequence accession numbers. The nucleotide sequences of the viral variants isolated 285 weeks after virus inoculation from diseased monkey 95-3 (Fig. 3) are available from GenBank (no. AF384152 through AF384159).

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