Analysis of Simian Immunodeficiency Virus Sequence Variation in Tissues of Rhesus Macaques with Simian AIDS

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One rhesus macaque displayed severe encephalomyelitis and another displayed severe enterocolitis following infection with molecularly cloned simian immunodeficiency virus (SIV) strain SIVmac239. Little or no free anti-SIV antibody developed in these two macaques, and they died relatively quickly (4 to 6 months) after infection. Manifestation of the tissue-specific disease in these macaques was associated with the emergence of variants with high replicative capacity for macrophages and primary infection of tissue macrophages. The nature of sequence variation in the central region (*vif*, *vpr*, and *vpx*), the *env* gene, and the *nef* long terminal repeat (LTR) region in brain, colon, and other tissues was examined to see whether specific genetic changes were associated with SIV replication in brain or gut. Sequence analysis revealed strong conservation of the intergenic central region, *nef*, and the LTR. However, analysis of *env* sequences in these two macaques and one other revealed significant, interesting patterns of sequence variation. (i) Changes in *env* that were found previously to contribute to the replicative ability of SIVmac for macrophages in culture were present in the tissues of these animals. (ii) The greatest variability was located in the regions between V1 and V2 and from "V3" through C3 in gp120, which are different in location from the variable regions observed previously in animals with strong antibody responses and long-term persistent infection. (iii) The predominant sequence change of D→N at position 385 in C3 is most surprising, since this change in both SIV and human immunodeficiency virus type 1 has been associated with dramatically diminished affinity for CD4 and replication in vitro. (iv) The nature of sequence changes at some positions (146, 178, 345, 385, and "V3") suggests that viral replication in brain and gut may be facilitated by specific sequence changes in *env* in addition to those that impart a general ability to replicate well in macrophages. These results demonstrate that complex selective pressures, including immune responses and varying cell and tissue specificity, can influence the nature of sequence changes in *env*.

In addition to immunodeficiency, gastrointestinal and neurological disorders frequently accompany infection with human immunodeficiency virus type 1 (HIV-1). Wasting, diarrhea, and cachexia are common sequelae of HIV infection, and gastrointestinal tissues from AIDS patients often show pathologic abnormalities in the absence of bacterial, viral, or parasitic infections (17). Neurological consequences not due to opportunistic infections or tumors, including AIDS encephalitis, neuropathy, meningitis, and myelopathy, are also frequent and devastating complications of HIV-1 infection (43, 48). Demonstration of HIV expression in the gut (15) and brain (19, 48, 57) of affected individuals and the susceptibility of neural cell lines (7, 11) and intestinal epithelial cell lines (1, 14, 33) to HIV infection in vitro argue for a primary causative role for HIV in the neurological and gastrointestinal disorders in AIDS.

HIV is able to infect not only T lymphocytes but also cells of the monocyte/macrophage lineage. In fact, infection of macrophages is a unifying feature of all animal lentiviruses (20), and the predominant cell type harboring HIV in tissues in many cases appears to be the macrophage, not the CD4⁺ lymphocyte (for a review, see reference 29). The principal targets of HIV in the brain appear to be macrophage/microglial cells (27, 55, 57). Most brain isolates exhibit the ability to

replicate in macrophages (8, 16, 28), and microglial cells are particularly susceptible to macrophage-competent strains of HIV-1 in vitro (54). The fundamental mechanisms underlying HIV-1 neuropathogenesis, however, remain to be elucidated.

The gastrointestinal tract is a putative site of entry for HIV. In the gut, HIV infects T lymphocytes and macrophages in the lamina propria (15); epithelial cells also appear to be targets in the gut (35). Interestingly, colonic epithelial cells can be more productively infected in vitro with macrophage-competent strains of HIV-1 than with T-cell-tropic strains (33). The identification of viral determinants governing HIV cell tropism is therefore important in understanding tissue-specific disease manifestations in AIDS. The genetic determinants for the ability of HIV-1 to replicate in macrophages have been mapped to the *env* gene by several investigators (21, 38, 49, 50, 56). Biologically and genetically different strains of HIV with distinct cell tropisms appear to coexist in various tissues within infected individuals, and this heterogeneity may contribute to the clinical and pathological variability observed in HIV-1-infected individuals.

Simian immunodeficiency viruses (SIVs) are nonhuman primate lentiviruses that closely parallel HIV in genomic organization, gene sequence, and biological properties (for a review, see reference 9). We have been studying rhesus macaques experimentally infected with pathogenic molecularly cloned SIV in order to identify the molecular determinants of

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cell and tissue tropism and the factors responsible for variability in disease manifestations. The infectious molecular clone SIVmac239 has been well characterized (34), and its genome has been completely sequenced (44). As with HIV infection in humans, the disease induced by SIVmac239 is heterogeneous in nature (23). Disease manifestations resulting from SIVmac239 infection include CD4 depletion, opportunistic infections, generalized lymphoid depletion, granulomatous encephalitis and pneumonia, and enterocolitis.

The infectious pathogenic SIVmac239 molecular clone replicates well in lymphocytes but poorly in macrophages in culture (23, 31). Pathologic findings in macaques infected with SIVmac239 vary from individual to individual, and it appears that tissue macrophage infection influences the nature of disease manifestations (10, 23). Many SIVmac239-infected animals die with AIDS, characterized by profound CD4⁺ lymphocyte depletion and opportunistic infections, but they do so without major infection of tissue macrophages. However, in some animals (approximately 30%), highly macrophage-competent variants of SIV evolve during the course of infection. The evolution of variants that are able to replicate in macrophages (macrophage-competent variants) is associated with specific pathologic manifestations, i.e., granulomatous encephalomyelitis and giant cell pneumonia, in which infected macrophages are characteristic of the lesion (10). SIV *env* sequences, like those of HIV-1, primarily determine the relative ability to replicate in the major target cells, i.e., macrophages and lymphocytes (2, 31). Thus, the appearance of macrophage-competent *env* variants in this system appears to be associated with tissue-specific disease manifestations.

Accumulating information suggests that the presence of macrophage-competent variants and tissue macrophage infection play a central role in tissue-specific disease manifestations in AIDS. One important question raised by these observations is the cause of individual variation in tissue-specific disease, whether macrophage-competent variants manifest their effects systematically or whether specific sequence changes over and above those that impart the ability to replicate in macrophages provide an enhanced ability of individual virus genotypes to replicate in selected tissues. As a first step toward addressing these issues, we report here the nature of sequence changes in different tissues of rhesus macaques that died after infection with cloned SIVmac239.

MATERIALS AND METHODS

Experimental infection of rhesus macaques. The SIVmac239 pathogenic molecular clone and its complete sequence have been described previously (34, 44) (GenBank accession number M33262). Two rhesus macaques (*Macaca mulatta*), Mm316-85 and Mm132-87, were inoculated intravenously with cloned SIVmac239 (10, 23). The macrophage-competent variant SIVmac316 was isolated previously from the lung of Mm316-85 (23). A pig-tailed macaque (*Macaca nemestrina*), Mn352-89, was inoculated intravenously with SIVmac316. Tissue specimens obtained from these animals at necropsy were fixed in 10% Formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological examination. Lymph node, lung, brain, gut, and peripheral blood lymphocytes from these animals were also frozen at -70°C and used as the source of genomic DNA.

PCR amplification. Total cellular genomic DNA was extracted from frozen tissues and used as templates in polymerase chain reaction (PCR) to amplify SIVmac sequences. Viral DNA sequences spanning the central region (*vif*, *vpx*, and *vpr*), full-length *env*, and the *nef* long terminal repeat (LTR)

region were separately amplified with the following sets of oligonucleotide primers: for the central region, nucleotides (nt) 5133 to 5159 (5'-CGCGCGGAATTCACATGATCAC TACAGAACAAAGAGATAC) and nt 6461 to 6426 (5'-ATGTG TTATAGCATGCTTCTAGAGGGCGGTATAGCT); for the *env* region, nt 6434 to 6471 (5'-CGCCCTCTAGAAGCATGC TATAACACATGCTATTGTAA) and nt 9244 to 9211 (5'-CTCACAAGAGAGTGAGCTCAAGCCCTTGTCTAAT); and for the *nef*-3' LTR region, nt 9214 to 9243 (5'-AGA CAAGGGCTTGAGCTCACTCTCTTGTGA) and nt 10279 to 10252 (5'-CGCGCGAATTCCTGCTAGGGATTTTCCTG CTTCGGTTTCC). Oligonucleotides corresponding to the 5' central region and the 3' right LTR contained an additional *Eco*RI recognition tag (underlined) for subsequent cloning. One microgram of genomic DNA was added to PCR mixes containing 1.5 mM MgCl₂, 200 μM each of the four dinucleoside triphosphates, 0.5 μM each oligonucleotide primer, 1 U of perfect match (Stratagene, La Jolla, Calif.), and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). PCR amplification was performed with an automated DNA Thermal Cycler (Perkin-Elmer Cetus) for 35 cycles with the following cycle profile: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1.5 min (3.5 min for full-length *env* amplification). An autoextension of 5 s was added to each cycle, and amplification was completed by a final extension at 72°C for 10 min. In order to minimize the effects of variation in each PCR, five PCR amplifications were performed with each sample. The resulting PCR products were pooled, and the amplified products were visualized by ethidium bromide staining.

Molecular cloning and sequencing. Amplified SIV DNA fragments were gel purified and subjected to restriction enzyme digestion for cloning. Sites for *Eco*RI, *Sph*I (nt 6450), and *Ssr*I (nt 9230) were used to clone different segments of the virus genome into the pBS(-) vector (Stratagene). Double-stranded plasmid clones were sequenced by the primer-directed dideoxy chain termination method with Sequenase (United States Biochemical Corp., Cleveland, Ohio) and [³⁵S]dATP.

Site-directed hybridization. The SIV *env* region corresponding to V3 of HIV-1 was amplified by PCR with a set of oligonucleotide primers (nt 7451 to 7480, 5'-AAAATAGA ACTTATATTTACTGGCATGGTA, and nt 7800 to 7771, 5'-GAAGTCTCCTCTGCAATTTGTCCACATGAA) from 1 pg of plasmid DNA or 1 μg of the tissue-derived genomic DNA. The amplified products (350 bp) were gel purified and treated with 0.1 volume of 3 M NaOH for 30 min at 65°C. The samples were then chilled in ice, and 1 volume of 2 M ammonium acetate (pH 7.0) was added. Approximately 200 ng of DNA was dotted onto a nitrocellulose membrane (0.2 μm; BA83; Schleicher & Schuell). A control oligonucleotide (nt 7589 to 7570, 5'-ACCAATCCAGACATAATGGT; calculated melting temperature [*T_m*], 56°C) and a mutagenic L334 oligonucleotide (5'-ATCATTGATTAGTTGTGAGTG; *T_m*, 56°C), made by a G to A mutation in the primer from nt 7614 to 7596 at position 7604 (underlined), were labeled with [³²P]ATP by using bacteriophage T4 polynucleotide kinase. Amplified DNA fragments immobilized onto nitrocellulose were hybridized with radioactive probes for 3 h at room temperature. The filter was washed with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at several temperatures for 5 min and then subjected to autoradiography for 3 h.

Nucleotide sequence accession numbers. The nucleotide sequences of the PCR-derived clones described in this report have been filed with GenBank under accession numbers L22790 through L22844.

TABLE 1. Association of macrophage (M ϕ) infection with tissue-specific disease manifestations

Animal	Virus	Tissue M ϕ infection	M ϕ -tropic virus isolation	Histopathology	PCR amplification ^a		
					Central	<i>env</i>	<i>nef</i> LTR
Mm316-85	SIVmac239	Yes	Yes (SIVmac316)	Brain: encephalomyelitis	+++*	+++*	+++*
				Lung: granulomatous pneumonia	+++*	+++*	+++*
				Lymph nodes: depletion	+++*	+++*	+++*
				Gut: mild acute inflammation	ND ^b	ND	ND
Mm132-87	SIVmac239	Yes	Yes (SIVmac132)	Brain: NSL ^c	—	—	—
				Lung: granulomatous pneumonia	+++	+++*	+++
				Lymph nodes: paracortical expansion	+++	+++*	+++
				Gut: gastroenteropathy	+++*	+++*	+++*
				PBMC: — ^d	+++	+++*	+++
Mn352-89	SIVmac316	ND	ND	Brain: encephalomyelitis	+++	+++*	ND
				Lung: granulomatous pneumonia	+++	+++*	ND
				Lymph nodes: paracortical expansion	+++	+++*	ND
				Gut: mild subacute inflammation	+	—	ND
				PBMC: —	+++	+++*	ND

^a The relative efficiencies of amplification of SIV sequences visualized by ethidium bromide staining on agarose gels are indicated. *, The amplified SIV DNAs were cloned and sequenced.

^b ND, not done.

^c NSL, no significant lesion.

^d —, not applicable.

RESULTS

Association of tissue macrophage infection with disease manifestations in SIVmac239-infected macaques. Rhesus macaque Mm316-85 died 168 days after infection with cloned SIVmac239, with granulomatous encephalomyelitis, granulomatous pneumonia, and generalized lymphoid depletion (Table 1) (23). Mm132-87 died 181 days after infection with cloned SIVmac239, with paracortical expansion of lymph nodes, granulomatous pneumonia, and severe SIV gastroenteropathy (Table 1) (10). Extensive tissue macrophage infection was previously demonstrated in both Mm316 and Mm132 (10). Macrophage-competent variants, called SIVmac316 and SIVmac132, were isolated previously from the lung and peripheral blood, respectively, of these animals (10, 23). The macrophage-competent variants replicate well in both the lymphocytes and macrophages of rhesus macaques in vitro. This contrasts markedly with parental SIVmac239, which replicates poorly in primary macrophage cultures from rhesus macaques. Thus, macrophage-competent variants evolved from the parental SIVmac239 during the course of infection in Mm316 and Mm132 in vivo. To study the importance of macrophage-competent variants for tissue-specific disease manifestations, we inoculated SIVmac316 into a pig-tailed macaque (Mn352-89). Mn352-89 died with AIDS after 4 months, with granulomatous pneumonia and encephalomyelitis (Table 1).

Total genomic DNA was prepared from the brain, lung, lymph nodes, colon, and peripheral blood mononuclear cells (PBMC) of Mm316, Mm132, and Mn352 and used as templates in PCR. Viral DNA sequences spanning the central region (*vif*, *vpx*, and *vpr*), *env*, and *nef* 3' LTR were tested separately for their ability to be amplified by PCR. Viral DNA sequences were specifically amplified from genomic DNA of tissues displaying histopathologic abnormalities (Table 1). The correlation of pathological findings with the specific amplification of SIV sequences from tissues suggests a direct role for SIV in the genesis of primary gastrointestinal and neurological disease. We selected Mm316 and Mm132 for initial detailed genetic analysis to investigate whether specific genetic changes

are associated with SIV replication in the gut and brain. PCR-derived viral DNA sequences spanning the central region, *env*, and *nef* LTR were cloned, and tissue-derived viral sequences were compared with parental SIVmac239 sequences.

Conservation of the central and *nef* LTR regions in vivo. The central region containing *vif*, *vpx*, and *vpr* was strongly conserved in clones from the brain, lymph nodes, and lung of Mm316 and in clones from the colon of Mm132. Nucleotide substitutions in this region averaged from 0.07 to 0.27% in the tissues examined from these two animals (Table 2). The *nef* LTR region was also well conserved in clones from the brain and lymph nodes of Mm316 and in clones from the colon of Mm132 (average nucleotide substitution, 0.27 to 0.54%) (Table 2). The parental SIVmac239 has an in-frame stop signal in the *nef* reading frame at codon 93 (44). Twelve of 13 *nef* LTR clones derived from the brain and lymph nodes of Mm316 had a reversion of a stop codon to tyrosine by a point mutation of A to C (Table 2). All of the colon-derived clones of Mm132 had a reversion of a stop codon to glutamic acid by a point mutation of T to G (Table 2). The importance of a full-length *nef* for vigorous virus replication in rhesus macaques and for pathogenesis has been demonstrated previously (24). The mutations in the central region and the *nef* LTR region were in general randomly distributed, and there were no predominant sequence changes that seemed to be associated with modified cell or tissue specificity (data not shown).

***env* sequence variation in brain of Mm316 and colon of Mm132.** Ten full-length *env* clones from the brain of Mm316 (316BSS) and five full-length *env* clones from the colon of Mm132 (132COL) were sequenced (Table 2 and Fig. 1). The average nucleotide substitution frequency relative to SIVmac239 was 0.54% for 316BSS clones and 0.73% for 132COL clones. Of the nucleotide substitutions, 82.4% in 316BSS and 76.8% in 132COL were nonsilent in that they changed the amino acid. Table 3 summarizes the frequencies of the various possible nucleotide substitutions in these 15 clones. High frequencies of G→A and A→G substitutions were observed, as has been noted previously with SIV (6, 22, 40) and HIV (18,

TABLE 2. Genetic changes in 3' half of SIVmac239 genome in vivo^a

Region	Origin of clone	No. of clones	Total no. of nucleotides	Total no. of base changes	Avg nucleotide substitution (%)	Codon 93 ^b of <i>nef</i>		% (no./total) nonsilent base changes ^c
						Change	No./total	
<i>vif-vpx-vpr</i> (1,086 bp)	Mm316, brain	5	5,430	10	0.18			
	Mm316, lymph nodes	4	4,344	3	0.07			
	Mm316, lung	5	5,430	10	0.18			
	Mm132, colon	5	5,430	15	0.27			
<i>nef</i> LTR (1,008 bp)	Mm316, brain	8	8,064	41	0.51	TAC (Tyr)	7/8	
	Mm316, lymph nodes	5	5,040	27	0.54	TAC (Tyr)	5/5	
	Mm132, colon	8	8,064	22	0.27	GAA (Glu)	8/8	
<i>env</i> (2,607 bp)	Mm316, brain	10	26,070	142	0.54			82.4 (117/142)
	Mm132, colon	5	13,035	95	0.73			76.8 (73/95)

^a Viral DNA sequences spanning the central region (*vif*, *vpx*, and *vpr*), *nef* LTR, and *env* were derived from the tissues of animals Mm316 and Mm132 by PCR and cloned. The nucleotide sequences of the PCR-derived clones were compared with those of parental SIVmac239 (44).

^b Reversion of in-frame stop codon TAA in the *nef* reading frame at codon 93 of parental SIVmac239 to coding codons in the tissue-derived clones.

^c Nucleotide substitutions that changed the amino acid in *env*.

52). A preference for C→T and T→C transitions was also noted in both sets of clones. Furthermore, the sites of nucleotide transitions seemed to occur preferentially within particular dinucleotides (Table 4). All G→A transitions in the 316BSS clones and 90% of the G→A transitions in the 132COL clones occurred within GA, GG, or AG dinucleotides. Similarly, the majority of A→G transitions occurred within AA, AG, or GA dinucleotides. Most of the C→T transitions occurred within CC or CT dinucleotides, and T→C transitions occurred predominantly within TT or TC dinucleotides. Thus, nucleotide substitutions, due largely to errors by reverse transcriptase (42, 46), appear to depend upon local sequences both 5' and 3' to the substituted nucleotide. Base substitution in the HIV-1 genome is also nonrandom and has also been reported to be affected by the local nucleotide sequence (12, 52).

As shown in Fig. 1, the positions of cysteine residues were completely conserved in all 15 *env* clones of 316BSS and 132COL. Eight of the 10 clones of 316BSS and four of the five clones of 132COL were completely open, without in-frame stop codons or frameshifts. In the three clones with multiple in-frame stop codons in *env* (316BSS1, 316BSS4, and 132COL3), none occurred in the region of the transmembrane protein (gp41), where they are seen in SIV adapted for growth in human cells (26). The *env*-encoded proteins gp120 and gp41 of SIVmac239 contain 27 potential sites for N-linked glycosylation (N-X-S or N-X-T, where N is asparagine, S is serine, T is threonine, and X can be any amino acid except proline and probably tryptophan). These sites were well conserved in the 15 clones; however, two potential sites for N-linked glycosylation were abolished by altered sequences at position 146 of the 132COL clones and at position 158 of the 316BSS and 132COL clones.

Unlike the random scattered changes observed in *vif*, *vpx*, *vpr*, and *nef*, the predicted amino acid substitutions within the 15 complete *env* clones occurred at discrete positions or regions (Fig. 1). In previous studies, five discrete variable regions, V1 (amino acids 115 to 142), V2 (188 to 202), V3 (371 to 376), V4 (404 to 426), and V5 (473 to 478), were identified in gp120 in rhesus macaques persistently infected with SIVmac239 (6) (Fig. 1). V1, V2, V4, and V5 of SIVmac239 correspond exactly to major regions of sequence variation in gp120 of HIV-1 (51). Interestingly, the variable positions in 316BSS and 132COL *env* clones did not correspond to variable regions identified in previous studies with SIVmac239 (6) or other SIV clones (22, 40). The rhesus macaques used in previous studies of SIV variation had strong antibody re-

sponses and remained persistently infected for prolonged periods prior to the development of AIDS. Sequence variation in Mm316 and Mm132, both of which died with a rapid course with little or no detectable free anti-SIV antibody, clustered at positions and in regions that were largely different from those found in previous studies (Fig. 1). One of the variable regions in the 316BSS and 132COL clones was located in the region between V1 and V2. Sequence changes of 382G→R, 385D→N, and 388V→A in both 316BSS and 132COL clones accumulated within a very narrow region corresponding to C3 in HIV-1. Also of interest is the fact that sequences corresponding to V3 of HIV-1 ("V3" in Fig. 1), which were not variable in previous SIV studies with SIVmac239 (6) and other SIV clones (22, 40), varied significantly in 316BSS and 132COL *env* clones (Fig. 1). Selective mutations of Leu-320 in 132COL and Pro-334 in 316BSS were observed within "V3".

The gp41 of SIVmac239 was relatively more conserved than gp120 in vivo. The proteolytic cleavage site between gp120 and gp41 (53) and the fusion domain located at the N terminus of gp41 (4) were strongly conserved in both 316BSS and 132COL clones. A variable region in gp41 (amino acids 626 to 660; V6 in Fig. 1) identified in rhesus macaques persistently infected with SIVmac239 (25) was conserved in 316BSS and 132COL clones. Clones 1, 2, and 7 were the only three from the brain of Mm316 in which Lys-573 was changed to threonine (Fig. 1). Interestingly, these were the only three clones from the brain of Mm316 in which proline 334 was not changed to arginine or leucine within V3. Similarly, only one clone from the colon of Mm132 (132COL3) showed the lysine 573 to threonine change, and this was the only clone which contained a Lys-320 to Val change within V3 (Fig. 1). This suggests that amino acids 320 and 334 in V3 and 573 in gp41 may be involved in some sort of cooperative interaction. The *env* reading frame overlaps *tat* exon 2, *rev* exon 2, and the N terminus of the *nef* coding region. A nucleotide change which converted Arg-751 to Gly in gp41 of 316BSS also resulted in changing a *rev* exon 2 amino acid from lysine to arginine. Otherwise, *tat* exon 2, *rev* exon 2, and the N terminus of the *nef* coding region were well conserved (data not shown).

Sequence changes at positions 67V→M and 382G→R, previously shown to contribute to replicative ability in macrophages (31), were present in all 15 clones (Fig. 1). Sequence changes at 176K→E and 573K→T, also previously shown to contribute to replicative ability in macrophages (31), were present in a portion of the clones from both animals. The 316BSS clones contained predominant sequence changes at some locations which were not detected in 132COL clones.

	10	20	30	40	50	60	70	80	90	100
	MGCLGNQLLIAILLLSVYGIYCTLYVTVFYGVPAWRNATIPLFCAATKNRDTWGTTCCLPDNGDYSEVALNVTFESFDANNIVTEQAIEDVWQLFETSIKP									
316BSS1env							M.			
316BSS2env		F.					M.			
316BSS3env			V.				M.			
316BSS4env							M.		R.	
316BSS5env		R.		V.			M.			
316BSS6env							M.	I.		
316BSS7env							M.			L.
316BSS8env							M.		I.	
316BSS9env							M.			
316BSS10env			V.				M.			
132COL1env				A.			M.			
132COL2env							M.			
132COL3env							M.			
132COL4env							M.			
132COL5env	I.	R.		R.			S.	M.		
	<div> <div><----- V 1 -----></div> <div><----- V 2 -----></div> </div>									
	110	120	130	140	150	160	170	180	190	200
	CVKLSPLCIITMRCKNSETDRWGLTKSITTTASTTSTTASAKVDMVNETSSCIAQDNCTGLEQEQMISCKFNMTGLKRDKKKEYNETWYSADLVCEQGNNT									
316BSS1env						A.		E.N.		G.
316BSS2env										
316BSS3env						A.				
316BSS4env		*						N.		
316BSS5env	A.				A.	A.		N.		
316BSS6env								N.		
316BSS7env						A.		E.N.		
316BSS8env										
316BSS9env						A.		N.		
316BSS10env		K.				A.		N.		
132COL1env					D.	A.		E.		
132COL2env					D.A.	A.		E.		
132COL3env					D.	A.		E.		
132COL4env					D.	A.		E.		
132COL5env		R.			D.	YA.		E.	K.	
	<div> <div>--></div> <div><----- V 3 -----></div> </div>									
	210	220	230	240	250	260	270	280	290	300
	GNESRCYMNHCNTSVIQESCCKHYWDATRFYCAPPGYALLRCNDTNYSGFMPKCSKVVSSTTRMMETQTSWFGFNGTRAENRTYIYWHGRDNRITIS									
316BSS1env										N.
316BSS2env										
316BSS3env		H.						S.		
316BSS4env						A.	A.			
316BSS5env						A.				
316BSS6env										N.
316BSS7env										
316BSS8env										
316BSS9env										
316BSS10env										
132COL1env										
132COL2env							A.			
132COL3env							A.			
132COL4env							A.			
132COL5env							A.			
	<div> <div> ----- "V 3" ----- </div> <div><----- V 3 -----></div> </div>									
	310	320	330	340	350	360	370	380	390	400
	LNKYNYLTMKRRPGNKTVLPVTIMSGLVFHSQPINDRPKQAWCFGGKWKDAIKEVKQTIIVKHPRYTGINNTDKINLTAPGGGDPEVTFMWINCRGEFL									
316BSS1env	I.							R..N..A.		
316BSS2env				N.				R..N..A.		
316BSS3env				R.				R..N..A.	S.	
316BSS4env				L.				R..N..A.		
316BSS5env				L.				R..N..A.		
316BSS6env		A.						R..N..A.		
316BSS7env	I.							R..N..A.		
316BSS8env				R.				R..N..A.		
316BSS9env				L.			S.	R..N..A.		
316BSS10env				L.				R..N..A.		
132COL1env		S.			R.			R..N..A.		
132COL2env	R.			N.	R.			R..N..A.		
132COL3env		V.			R.	*		R..N..A.		
132COL4env		S.			R.			R..N..A.		
132COL5env		S.			R.			R..N..A.		
	<div> <div><----- V 4 -----></div> <div><----- V 5 -----></div> </div>									
	410	420	430	440	450	460	470	480	490	500
	YCKMNVFLNWVEDRNTANQKPKQHKRNVVPCIRIQIINTWHKVGKNVYLPREGDLTCNSTVTSLIANIDWIDGQNTITMSAEVAELRLELDYKLV									
316BSS1env		S.								
316BSS2env		S.		Y.						
316BSS3env		S.								
316BSS4env	*	S.								
316BSS5env		S.								
316BSS6env		S.								
316BSS7env		S.								
316BSS8env		S.								
316BSS9env										
316BSS10env		S.								
132COL1env									G.	
132COL2env		L.							G.	
132COL3env		L.							G.	
132COL4env									G.	
132COL5env		L.

FIG. 1. Amino acid variations in Mm316 brain- and Mm132 colon-derived *env* clones. Predicted amino acid sequences of the Mm316 brain (316BSS) and Mm132 colon (132COL) derived *env* clones were aligned with those of SIVmac239 (44) (top line). Dots represent identical amino acids, and asterisks indicate a translational in-frame stop codon. Variable regions V1 to V6, previously identified in rhesus macaques persistently infected with SIVmac239 (6, 26), are shown. The location of SIV regions corresponding to the V3 cysteine loop of HIV-1 are indicated by "V3." The proteolytic cleavage site between gp120 and gp41 (53) is shown.

These included 178D→N, 334P→L or R, and 421P→S. Conversely, the 132COL clones contained predominant sequence changes not detected in 316BSS clones. These included 146N→D, 271T→A, 320L→S or V, 345W→R, 421P→L, 488E→G, and 851D→N or V.

Additional *env* sequence analysis. In order to analyze in greater detail the association of unique sequence changes with SIV replication in specific tissues, additional *env* sequence information was derived. *env* sequences corresponding to the variable regions in the 316BSS and 132COL clones (amino

TABLE 3. Nucleotide substitution frequencies of 316BSS and 132COL *env* clones

Clone origin	No. (%) of nucleotide substitutions ^a											
	A			G			C			T		
	G	C	T	A	C	T	A	G	T	A	G	C
316BSS	35 (24.6)	4 (2.8)	2 (1.4)	59 (41.5)	0 (0)	0 (0)	1 (0.7)	2 (1.4)	24 (16.9)	0 (0)	2 (1.4)	13 (9.2)
132COL	36 (37.9)	1 (1.1)	3 (3.2)	32 (33.7)	0 (0)	3 (3.2)	0 (0)	0 (0)	3 (3.2)	2 (2.1)	3 (3.2)	12 (12.6)

^a The number and percentage of mutational events (from a nucleotide in the upper row to one in the lower row) in 10 *env* clones from the brain of Mm316 (316BSS) and five *env* clones from the colon of Mm132 (132COL) are shown. The high frequency of A to G and G to A substitutions and preference for C to T and T to C transitions are evident in both set of clones.

acids 132 to 232 and 283 to 486) were derived from the lymph nodes and lung of Mm316, from the lymph nodes, lung, and PBMC of Mm132, and from the brain, lymph nodes, lung, and PBMC of Mn352 that died with AIDS following infection with SIVmac316 (Fig. 2A and B). If specific sequence changes are indeed associated with enhanced ability to replicate in specific tissues, two types of results might be anticipated. First, selective sequence changes may be present in different tissues of the same animal. Alternatively, sequence changes that arise in a single animal may allow replication in macrophages in many tissues, but these changes may preferentially facilitate virus replication and disease manifestation in one tissue relative to others in that animal.

Most of the changes observed in the brain of Mm316 were also observed to a similar extent in the lymph nodes and lung of the same animal (Fig. 2). However, the change of 178D→N, observed in 7 of 10 clones from the brain, was observed in only two of nine clones from the lymph nodes and one of five clones from the lung (Fig. 2A). Similarly, the change of 334P→L, observed in 5 of 10 clones from the brain, was not observed in any clones from lymph nodes or lung from the same animal (Fig. 2B). Pro-334 was changed to Arg, Ser, or Thr in clones derived from the lymph nodes and lung from Mm316. Since only a limited number of clones were sequenced, mutation-specific hybridization was used to examine the brain specificity of the 334P→L change. Fragments of 350 bp spanning V3 were amplified from genomic DNA of the lymph nodes and brain of

Mm316, from plasmid DNA of 316BSS, and from parental SIVmac239. Hybridization with an L334 oligonucleotide, having a point mutation encoding leucine at position 334, gave strong signals with PCR products from the brain of Mm316 and with 316BSS4, but not with PCR products from the lymph nodes of Mm316 or with parental SIVmac239 (Fig. 3). These results indicate that the 334P→L change in V3 was a predominant and selective substitution in the brain of Mm316.

Pig-tailed macaque Mn352 displayed encephalomyelitis and giant cell pneumonia after infection with macrophage-tropic SIVmac316 (Table 1). Analysis of *env* clones derived from Mn352 corresponding to amino acids 133 to 232 and 283 to 486 revealed sequence changes at positions similar to those seen in Mm316. Only a few amino acid changes unique to Mn352 were observed in these regions. These include 217Q→K, 383G→R, and 444V→I in a portion of the clones (Fig. 2). The 178D→N change was observed in four of eight clones from the brain but was not in any other tissues of Mn352. 178D→N also predominated in the brain of Mm316 but was a minority sequence in other tissues from this animal (Fig. 2A; see above). 178D→N was not observed in any of the six clones analyzed from the SIVmac316 virus stock (31). A change of 334P→L in V3 was not observed in any of the clones from Mn352. Therefore, the change of 334P→L, seen in 5 of 10 clones from the brain of Mm316, is not specifically associated with SIV in encephalitic brains. However, a different change at the same position, 334P→S, was observed in a preponderance of clones from Mn352 (Fig. 2B).

Most of the changes that were observed in the colon of Mm132 were also observed to a similar extent in the lymph nodes, lung, and PBMC of the same animal (Fig. 2). Thus, specific sequence preferences were not observed within individual tissues of Mm132. In contrast to Mm316- and Mn352-derived clones, Leu-320 in V3 strongly converted to Ser in all tissue-derived clones from Mm132. Substitutions 146N→D and 345W→R were also observed specifically in tissues from Mm132 but not from the other two animals (Fig. 2).

Table 5 summarizes the major amino acid changes in gp120 of the tissue-derived *env* clones and those of macrophage-competent SIVmac316 and SIVmac132 (31) isolated in culture. The major amino acid changes in gp120 of SIVmac316 and SIVmac132 were present in the tissue-derived clones from Mm316 and Mm132, respectively (Table 5). However, some sequence changes that have not been associated previously with cell culture-isolated SIVmac316 and SIVmac132 predominated in clones from the tissues of these animals. Furthermore, in contrast to the variation in V3 sequences in the tissue-derived clones, the V3 sequences of SIVmac316 and SIVmac132 have been found to be identical to those of parental SIVmac239 (31) (Table 5).

The other striking feature of the tissue-derived *env* clones is the change at position 385 from D to N in the sequence

TABLE 4. Preferential sites for nucleotide substitution in 316BSS and 132COL *env* clones^a

Mutation	Site	316BSS clones		132COL clones	
		No. of transitions	% of transitions	No. of transitions	% of transitions
G→A	GpA	30	51	11	34
	GpG	19	32	15	47
	ApG	10	17	3	9
	Total		100		90
A→G	ApA	27	77	24	66
	ApA, ApG, or GpA	31	88	31	86
C→T	CpC	17	70	3	100
	CpT	3	12		
	Total		82		
T→C	TpT	8	62	11	92
	TpC	3	23	1	8
	Total		85		100

^a Preferential neighboring nucleotides are shown for the sites of G→A, A→G, C→T, and T→C transitions that are defined as predominant mutational events in 316BSS and 132COL *env* clones in Table 3.

(A)

	140	150	160	170	180	190	200	210	220	230
	TTSSTTASAKVDMVNETSSCIAQDNCTGLEBQEQMISCKFNMTGL KRDKKKEYNETWYSADLVCEQGNNTGNESRCYMNHCNTSVIQESCDKHYWDAIRFRY									
Mm316										
316LSS1env			I							
316LSS2env			A							
316LSS3env			A							
316LSS4env		N	A	K	KN	K	*	K	K	I
316LSS5env			A	K	E	N		I		
316LSS6env			A							
316LSS7env			A						H	
316LSS8env			A						H	
316LSS9env			A						I	
316LSS10env			A						L	
316LUN1env					E					
316LUN2env					E					
316LUN3env			A							
316LUN4env		I	A		E	N				
316LUN5env			A		E			D		
Mn352										
352BSS1env			A		N				K	
352BSS2env			A		K	K			K	
352BSS3env			A		N	G				
352BSS4env			A		N					
352BSS5env			A		X	K			K	
352BSS6env			A						Q	
352BSS7env			A		K				K	
352BSS8env			A		N					
352LSS1env			A		K				K	
352LSS2env		A	A							
352LSS3env		A	A		K				K	
352LSS4env			A		K				K	
352LUN1env										
352LUN2env		S	A							
352LUN3env										
352LUN4env										
352PBL4env		A	A	R						
352PBL1env			A		K				K	
352PBL2env		A	A		K				K	
352PBL3env		A	A		K				K	
352PBL5env		E	A		K				K	
Mm132										
132LSS1env		D	A	I	E					
132LSS2env		D	A	I	E					
132LSS3env		D	A		E	E	A			
132LSS4env		D		I	E			K		
132LUN3env		D	A							
132LUN2env		D	A	V						
132LUN5env					E					
132LUN4env		D	A		E					
132LUN1env		D	A		E	R		K		
132PBL1env		D	A		E					
132PBL4env		D	A		E					
132PBL5env		D	A		E					
132PBL3env		D	A		E			K		
132PBL2env		D	A		G					

FIG. 2. *env*-encoded amino acid changes in tissues of macaques that died with specific disease manifestations. Predicted amino acid sequences from *env* amino acids 133 to 232 (A) and 283 to 486 (B) of *env* clones derived from the tissues of Mm316, Mn352, and Mm132 were aligned with those of parental SIVmac239. BSS, brain; LSS, lymph nodes; LUN, lung; PBL, peripheral blood lymphocytes (source of DNA for PCR amplification and cloning). The layout of this figure is identical to that of Fig. 1 except that X represents a frameshift mutation.

GGDPE (Fig. 1 and 2B), which is highly conserved in both SIV (amino acids 383 to 387) and HIV-1 (366 to 370). Olshevsky et al. showed that mutations of D to N at this location in HIV-1 resulted in dramatic reduction in CD4 binding ability without affecting the processing and cell association of gp120 (39). A change of D to N at this location in SIVmac239 also results in dramatic reduction in CD4 binding and the ability of virus to replicate in human T-cell lines (32). What is most notable is the high percentage of Mm316-, Mm132-, and Mn352-derived *env* clones with an amino acid change at this position (Table 5). A remarkable 55 of 64 clones (85%) from a variety of tissues of three different macaques had a change at position 385, and 45 of these clones had a D to N conversion.

DISCUSSION

In this study, we analyzed the evolution of sequence changes in the SIV genome by cloning directly from tissues of rhesus macaques that died with neurological or gastrointestinal disease after infection with cloned SIVmac239. The ability to amplify SIV sequences specifically from affected tissues suggests that SIV plays a direct role in the tissue-specific disease manifestations. In contrast to the strong conservation of the central region (*vif*, *vpx*, and *vpr*), *nef* coding region, and 3' LTR region, sequences in gp120 showed striking variations in the affected tissues. This suggests an important role for the *env* genetic changes in the tissue-specific replication and disease

(B)

(b/	290	300	310	320	330	340	350	360	370	380	390				
	-----"v3"-----														
	<--v3-->														
239	ENRTYIYWHGRDNR	TIISLNKY	NLTMTK	RRPGNK	IVLPVT	IMSGLV	FHSQPI	NDRPKQ	AWCWF	GGKWKDAI	KEVKQTIVKH	PRYTGTINNTDKINL	TAPGGG	DEPVT	F
Mm316															
316LSS1env							R							R	N
316LSS2env							S	N						R	N
316LSS3env							R			N				R	G
316LSS4env		KN		I										R	N
316LSS5env															
316LSS7env							R							R	N
316LSS9env		N												R	N
316LSS8env							R			SI				R	G
316LSS6env		N		I										R	N
316LUN1env														R	
316LUN2env							T	K						R	
316LUN3env					S		S		R					R	N
316LUN4env		N		I										R	N
316LUN5env							S							R	
Mn352															
352BSS1env		N		I	K		S							R	N
352BSS2env		N		I			S	N						RR	G
352BSS3env														R	N
352BSS4env														R	N
352BSS5env							S							RR	K
352BSS6env		N					S	N						R	G
352BSS7env							S	N						RR	N
352BSS8env														R	N
352LSS1env		N		I	K		S							RR	N
352LSS2env		N		I			S	N						RR	N
352LSS3env		N		I			S	N						RR	N
352LSS4env				I			S	N						RR	N
352LUN1env															
352LUN2env		N					S							RR	N
352LUN3env															
352LUN4env															
352PBLenv4		N		I			S							R	G
352PBLenv2		N		I			S	N						R	G
352PBLenv1		N					S							RR	N
352PBLenv3		N		I			S							RR	N
352PBLenv5		K					S							R	G
Mm132															
132LSS1env					S			R	E					R	N
132LSS2env					S			R	E					R	N
132LSS3env					S			R						R	N
132LSS4env					S			R						RR	N
132LUNenv5														R	
132LUNenv4					S			R						R	N
132LUNenv3					S			K	R					R	N
132LUNenv2					R			K	R					R	N
132LUNenv1					S			R	E					R	H
132PBLenv1		K						K	R					R	
132PBLenv3							N		R					R	N
132PBLenv4					S			R						RR	N
132PBLenv2					S	P		K	R					R	N
132PBLenv5					S			R						R	H

FIG. 2—Continued.

manifestations of SIV in these animals. The genetic conservation of LTR sequences in tissue and the importance of *env* sequences for the pathogenesis of SIV_{mmPBj} have also been reported (36, 37).

Infection of macrophages was clearly identified in the tissues of Mm316 and Mm132, and macrophage-competent variants SIV_{mac316} and SIV_{mac132} were isolated from these animals. Major amino acid changes in gp120 of SIV_{mac316} and SIV_{mac132} were also present in the tissue-derived *env* clones from Mm316 and Mm132 (Table 5). Changes of 67V→M, 176K→E, 382G→R, and 573K→T, which together dramatically enhance the ability to replicate in macrophages (31), were observed in various degrees in the tissues of these animals. These data indicate that the emergence of SIV macrophage-competent *env* variants is associated with extensive virus replication in and histopathologic changes to both the gut and brain.

Although giant cell diseases were a common feature in Mm316, Mm132, and Mn352, the tissue specificity of the disease manifestations was quite different. Mm316 and Mn352 displayed severe neurological disorders, but only mild inflammation was observed in the gastrointestinal tracts of these animals. In contrast, Mm132 showed very severe gastroenteropathy, but no histologic abnormalities were observed in the brain. These results indicate that virus replication in macrophages does not necessarily manifest equally in all tissues of an individual animal. Although there were no absolute associations of sequence changes with tissue tropism in the current study, the nature of sequence changes at some positions is consistent with facilitation of virus replication in specific tissues, and this could contribute to manifestations of neurological or gastrointestinal disease. A change of 178D→N was observed in 7 of 10 clones from the brain of Mm316 and four

	400	410	420	430	440	450	460	470	480
	←-----V 4-----→								
	←-V5-→								
239	MW	TN	CR	GE	FL	YCK	MN	WF	LN
Mm316	W	V	E	D	R	N	T	A	N
316LSS1env						S			
316LSS2env						S			
316LSS3env						S			
316LSS4env						S			
316LSS5env						S			
316LSS7env						S			
316LSS9env						S			
316LSS8env						S			
316LSS6env						S			
316LUN1env									
316LUN2env									
316LUN3env						SR			
316LUN4env						S			
316LUN5env						S			
Mn352									
352BSS1env									
352BSS2env									
352BSS3env									
352BSS4env									
352BSS5env									
352BSS6env									
352BSS7env									
352BSS8env									
352LSS1env									
352LSS2env									
352LSS3env									
352LSS4env									
352LUNenv1									
352LUNenv2									
352LUNenv3									
352LUNenv4									
352PBLenv4									
352PBLenv2									
352PBLenv1									
352PBLenv3									
352PBLenv5									
Mm132									
132LSS1env									
132LSS2env									
132LSS3env									
132LSS4env									
132LUNenv5									
132LUNenv4									
132LUNenv3									
132LUNenv2									
132LUNenv1									
132PBLenv1									
132PBLenv3									
132PBLenv4									
132PBLenv2									
132PBLenv5									

FIG. 2—Continued.

of eight clones from the brain of Mn352. This change was not observed in any of the 19 clones from Mm132, which did not have brain disease; in any of the 13 clones from the lymph nodes, lung, and PBMC of Mn352; or in 11 of 14 clones from the lymph nodes and lung of Mm316 (Table 5). A change of P to L, S, or T at position 334 in V3 was observed in 30 of 45 clones from Mm316 and Mn352, which had brain disease, but in 0 of 19 clones from Mm132, which did not have brain disease (Table 5). Interestingly, brain-specific sequence changes in V3 of HIV-1 have also been reported in pediatric AIDS patients (13). A change of 146N→D and 345W→R was observed in 19 of 19 clones from Mm132, which had severe gut disease, but in 0 of 45 clones from Mm316 and Mn352 (Table 5). Similarly, a change of L to S, V, or R at position 320 in V3 was observed in 15 of 19 clones from Mm132 but was not observed in the other animals. Therefore, the emergence of specific changes in the viral *env* sequence may be one means by which replication

in specific tissues is facilitated and may contribute to tissue-specific disease manifestations.

The nature and location of sequence changes documented in the present study are very different from those described in previous studies of SIV variation with SIVmac239 (6, 25) (Fig. 1) or other clones of SIV (22, 40). The sequence variations observed in this study accumulated predominantly in the regions between V1 and V2 and from V3 through C3. The differences in the locations of sequence variations in the present study are likely due to differences in the nature of the selective pressure driving genetic change. The macaques used in the previous studies had strong, stable immune responses and remained persistently infected for a prolonged period before the development of AIDS. A strong immune response is likely to be a major selecting force driving change in the persistently infected macaques (5). Animals Mm316, Mm132, and Mn352 showed rapid disease progression and died without

FIG. 3. Site-directed hybridization to detect leucine at amino acid position 334 in SIV present in the brain of Mm316. DNA fragments of SIV corresponding to the V3 cysteine loop of HIV-1 (nt 7451 to 7800 of SIVmac239, 350 bp) were amplified by PCR from (lanes 1) parental SIVmac239SpE plasmid (23), (lanes 2) Mm316 brain-derived *env* clone 316BSS4, (lanes 3) genomic DNA from the lymph nodes of Mm316, and (lanes 4) genomic DNA from the brain of Mm316. Amplified DNA (200 ng) was dot-blotted onto nitrocellulose filters and hybridized with ³²P-5'-end-labeled (A) control oligonucleotide (*T_m*, 56°C) or (B) L334 oligonucleotide (*T_m*, 56°C). The filters were washed with 2× SSC at the indicated temperatures for 5 min and then subjected to autoradiography for 3 h.

ever mounting a significant, detectable level of free anti-SIV antibodies. Therefore, in this study, selective pressures other than a strong immune response likely contributed to a very different pattern of *env* sequence variation.

One of the interesting features of the present study is the nature of sequence variation in V3. In the presence of a strong host immune response, the sequences in V3 in SIVmac239 were previously found to be quite conserved in persistently infected animals (6). The V3 sequences of SIVsm and SIVmne have also been reported to be well conserved in vivo (22, 40). Mm316, Mn352, and Mm132 in this study had very weak antibody responses, and the *env* clones obtained from them revealed extensive changes within the V3 sequences. The V3 of HIV-2, which is more closely related to SIV than HIV-1, varies among field isolates (3). It therefore seems that the V3 of SIV and HIV-2 may not be conserved, as previously thought, and may vary to a comparable degree from that observed in HIV-1.

The amino acid substitutions in V3 of the tissue-derived *env* clones in these studies were not random mutations; apparent selective pressure for a particular amino acid residue within V3 occurred at Pro-334 in Mm316 and Mn352 and at Leu-320 in Mm132 during the relatively short infectious course (4 to 6 months). V3 of HIV-1 is important not only as a neutralizing epitope (47), but also in determining cell tropism (21, 50). It has been reported that the V3 of SIV and HIV-2 do not constitute linear neutralizing epitopes, like that of HIV-1 (45). Recent work from our laboratories, however, indicates that SIVmac V3 is similar to HIV-1 V3 in determining cell tropism at the level of viral entry (24a, 30, 32). In contrast to the tissue-derived *env* clones, the V3 sequences of macrophage-competent SIVmac316 and SIVmac132 isolated in culture from Mm316 and Mm132 were identical to those of parental SIVmac239 (31) (Table 5). This finding suggests that SIV variants with mutations in V3 in vivo may have been counter-selected by in vitro culture. Thus, the sequence variation in SIVmac V3 observed in the present study may be driven not by immune responses but by the enhanced ability to replicate in different cell types in vivo.

The sequence change at position 385 from D to N in 45 of 64 clones examined from the three animals is very surprising and very difficult to understand at present. The sequence GGDPE in C3 is highly conserved in laboratory isolates of SIV (amino acids 383 to 387) and HIV-1 (366 to 370). Olshevsky et al. (39) showed that mutation of D to N at this location in HIV-1 results in dramatic reduction in CD4 binding ability without affecting the processing or cell association of gp120. Morrison et al. have also shown that a change of D to N at this location in SIVmac239 results in dramatic reduction in CD4 binding and the ability of virus to replicate in vitro (32). Infection of T lymphocytes and monocytes/macrophages by HIV-1 and SIV appears to be predominantly a CD4-mediated event (31, 41). Therefore, one possibility is that there may be a compensating change or changes elsewhere in *env* that would allow CD4 binding. Morrison et al. recently showed that a change in SIV V3 can compensate for a mutation in C4 to allow SIV entry and virus replication (32). The association of V3 sequence changes at position 320 in 132COL clones and at position 334

TABLE 5. Summary of *env*-encoded amino acid changes in clones from Mm316, Mm132, and Mn352^a

		a.a.133-232							311-344 c-v3-c												a.a. 283-486								
SIVmac239			146 N	158 T	165 H	176 K	177 R	178 D	217 Q	294 D	309 H	320 L	334 P	345 U	347 G	382 G	383 G	385 D	388 U	421 P	422 K	444 U	471 D	476 H					
Mm316	Brain	(10)	A ₆		e ₂		N ₇		h ₁	n ₂	i ₂	L ₅ f ₂		R ₁₀		N ₁₀		A ₆	S ₉										
	Lym. node	(9)	S ₁	A ₇ i ₁		e ₁		k ₁	n ₂	h ₃ l ₁	n ₃	i ₂	r ₄ S ₁		R ₈		N ₆ g ₂		A ₈	S ₈		n ₁							
	Lung	(5)	a ₂		E ₄		n ₁		n ₁												i ₁	n ₁							
SIVmac316			E							R																			
Mn352	Brain	(8)	A ₈				k ₃		N ₄	K ₄	n ₃	i ₂	S ₅		R ₈		r ₃	N ₅ g ₂ k ₁				i ₁	n ₁						
	Lym. node	(4)	A ₄				K ₃		K ₃		N ₃	I ₄	S ₄		R ₄		R ₄	N ₄	A ₂		T ₃		I ₄						
	Lung	(4)	S ₁	a ₁								n ₁		S ₁		r ₁		r ₁	n ₁				n ₁						
	PBMC	(5)	A ₅				K ₄		K ₄		N ₄	I ₃	S ₅		R ₅		r ₂	G ₃ n ₂				I ₅							
Mm132	Lym. node	(4)	D ₄	A ₃	I ₃	E ₄														S ₄	R ₄	E ₂	R ₃	r ₂	N ₄	S ₃		n ₁	N ₄
	PBMC	(5)	D ₅	A ₅			E ₄	g ₁													S ₃	R ₅	R ₄		r ₂	N ₃ h ₁	L ₃		
	Colon	(5)	D ₅	A ₅			E ₅													S ₃ v ₁	R ₅	R ₅				N ₅	a ₂	L ₃	
	Lung	(5)	D ₄	A ₄			E ₃													S ₃ r ₁	R ₄	e ₁	R ₅			N ₃ h ₁	I ₂ S ₁	n ₁	n ₁
	SIVmac132			D	A	E														E R						D			

^a Major and minor amino acid (a.a.) changes are shown in boldface capitals and lightface lowercase letters, respectively. The number of clones with each variation is shown as a subscript (the number of clones examined is shown in parentheses). The SIV *env* region corresponding to V3 of HIV-1 is indicated by "V3." The consensus changes in SIVmac316 and SIVmac132, isolated in cell cultures (31), are also shown.

in 316BSS clones with amino acid 573 in gp41 in the present study could also be due to cooperative effects. Alternatively, the changes at position 385 in SIV in tissues may allow entry into macrophages, at least as they exist in some tissues.

Thus, complex selective pressures, including immune responses, varying cell and tissue specificity, in vitro selection, and replicative capacity, appear to influence the nature of sequence changes observed in V3 and other regions of the SIV envelope. Further studies are needed, however, to demonstrate which sequence changes in *env* actually facilitate virus replication in particular cell types in vivo and contribute to tissue-specific disease manifestations.

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