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Unique Pattern of Convergent Envelope Evolution in Simian Immunodeficiency Virus-Infected Rapid Progressor Macaques: Association with CD4-Independent Usage of CCR5

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The rate of disease development in simian immunodeficiency virus (SIV) infection of macaques varies considerably among individual macaques. While the majority of macaques inoculated with pathogenic SIV develop AIDS within a period of 1 to 2 years, a minority exhibit a rapid disease course characterized by absence or transience of humoral and cellular immune responses and high levels of virus replication with widespread dissemination of SIV in macrophages and multinucleated giant cells. The goal of this study was to examine viral evolution in three SIVsmE543-3-inoculated rapid progressors to determine the contribution of viral evolution to the development of rapid disease and the effect of the absence of immune pressure upon viral evolution. PCR was used to amplify and clone the entire SIV genome from tissues collected at necropsy, and the course of viral evolution was assessed by *env* sequences cloned from sequential plasma samples of one rapid progressor (RP) macaque. The majority of sequence changes in RP macaques occurred in the envelope gene. Substitutions were observed in all three animals at specific conserved residues in envelope, including loss of a glycosylation site in V1/V2, a D-to-N/V substitution in a highly conserved GDPE motif, and a P-to-V/H/T substitution in the V3 loop analog. A cell-cell fusion assay revealed that representative *env* clones utilized CCR5 as a coreceptor, independent of CD4. The selection of specific substitutions in envelope in RP macaques suggests novel selection pressures on virus in such animals and suggests that viral variants that evolve in these animals may play a role in disease progression.

Human immunodeficiency virus type 1 (HIV-1) infection of humans is a fatal disease in the vast majority of untreated patients, with a median survival of about 10 years from the time of diagnosis. However, the disease course in HIV-infected patients is highly variable, ranging from long-term asymptomatic survival for more than 15 years (9, 16, 56) to rapid progression to AIDS within 1 or 2 years of infection (41, 45, 46). Persistent replication of virus is observed throughout infection (18, 57, 69). The level at which plasma viremia stabilizes following primary HIV infection is a highly significant prognostic indicator of the subsequent course of disease (44, 52), suggesting that host immune mechanisms in the early period after seroconversion are critical in the control of viremia. Indeed, the development of cytotoxic T cells specific for HIV occurs concurrently with a decrease in primary plasma viremia consistent with a role of $CD8^+$ cytotoxic T lymphocytes in controlling virus replication (39, 49). The reasons behind nonprogression are unclear but encompass both host and viral factors. Host factors that influence disease progression include deletions in the chemokine coreceptor gene (CCR5) (16, 67), strength of the cytotoxic T-lymphocyte response (49, 52, 65), strength of the antibody response (42), and major histocompatibility complex class I haplotype (10, 22). In addition, factors such as the biologic phenotype of the infecting virus, coreceptor usage, or attenuating mutations such as *nef* gene deletions in the infecting viral strain may also influence the rate of disease progression (15, 19). The rate of evolution of the HIV-1 envelope varies depending upon the rate of disease progression for the patient (44). The viral strains, dose, and route of infection are highly variable among HIV-infected patients, making the analysis of the contributions of host mechanisms to differences in disease progression complex.

The simian immunodeficiency virus (SIV) macaque model is well established for the study of potential correlates of disease progression and virus evolution in vivo. The median survival of SIV-infected macaques is considerably shorter than for HIV-infected humans, ranging from 1 to 2 years depending upon the strain of virus (2, 26). Like HIV-1 infected humans, SIV-infected macaques exhibit a variable disease course even when inoculated with a common molecularly cloned virus (23, 24, 31). Although the majority of SIV-infected macaques develop AIDS within 1 to 3 years of inoculation, a small percentage of infected animals exhibit a rapid disease progression phenotype (70). This syndrome is characterized by the transience or lack of SIV-specific humoral immune responses and persistent plasma antigenemia. These animals generally succumb to a wasting disease within 3 to 6 months of inoculation (25, 26, 68, 71). As observed for humans infected with HIV-1, the postseroconversion viral load or viral load set-point is also a strong predictor of disease progression in the SIV/macaque model

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(25, 68). The rate of development of viremia and the peak levels during primary viremia also appear to correlate with disease progression (40, 51, 66). Macaques that progress rapidly characteristically exhibit persistent high levels of plasma viremia (often $>10^8$ copies/ml).

SIV occurs in vivo as a genetically diverse quasispecies that replicates at a high rate and thus causes considerable turnover of virus and infected cells (30). This phenomenon allows for emergence and accumulation of genetic diversity in the quasispecies. Studies of genetic variation are therefore complicated by the complexity of the quasispecies in the virus inoculum and variable replicative rates within the quasispecies. The use of molecularly cloned virus allows for the study of the role of virus evolution in disease progression without the confounding factors stated previously. Genetic variation in the virus population is controlled by factors such as the number of replication cycles, the mutation rate due to the error-prone nature of reverse transcriptase, and selective pressures, such as the host immune responses. Variable regions of SIV have been previously defined by the study of the virus with animals that progressed to AIDS in the face of persistent antiviral immunity (7, 29). These studies have identified four discrete variable regions within the SIV envelope, V1, V2, V4, and V5. The region analogous to V3 of HIV-1 was generally highly conserved in SIV-infected macaques. Because these regions were defined for animals with robust immune responses, particularly neutralizing antibody responses, evolution of these regions is likely to be the result of viral mechanisms of immune evasion. The primary regions subject to genetic variation during the course of SIV infection in macaques are limited to the V1 and V4 regions of the envelope glycoprotein (3, 20, 55). Variation in the V1 region is characterized by substitutions and short, in-frame deletions and insertions. A few studies of viral evolution have included macaques that developed diseases associated with the emergence of macrophage-tropic variants of SIV, i.e., encephalitis. Envelope clones from such animals revealed an unusual distribution of substitutions in previously defined conserved regions, such as a D385N change that has been reported to be associated with a diminished affinity for CD4 (53). Previous studies of virus in lymphoid tissues of macaques that progressed rapidly with encephalitis after inoculation with uncloned SIVsm strains also revealed similar variability in the V3 loop analog (8, 24, 27).

The goal of the present study was to define patterns of virus evolution in the absence of host immune response in animals that exhibited a rapid disease phenotype compared to those that progressed normally to AIDS. To eliminate the possibility of selection of variants from the inoculating virus, these studies focused on macaques inoculated with the pathogenic, molecularly cloned SIVsmE543-3 virus strain. The data presented represent a comprehensive analysis of viral genetic variation in tissues of three rapid progressor (RP) macaques inoculated with the SIVsmE543-3 molecular clone.

MATERIALS AND METHODS

Virus and animals. Two pig-tailed macaques (Pt573 and Pt583) and one rhesus macaque (Rh445) were inoculated with the well-characterized SIVsmE543-3 molecular clone as part of other ongoing studies. Animals were housed in accordance with the NRC Guide for the Care and Use of Laboratory Animals (14). Each of these animals fit the criteria of rapid progression, lacking measurable SIV-specific antibody responses and exhibiting persistent antigenemia and increasing levels of viral RNA in plasma. The in vivo pathogenesis of SIVsmE543-3 in these animals has been described in detail previously (20, 21). Tissues were collected at necropsy following deep anesthesia and saline perfusion to remove residual blood in tissues, and the samples were frozen and stored in liquid nitrogen until use. Sequential EDTA-anticoagulated plasma samples were collected from H445, H444, H455, and H460 and stored in liquid nitrogen until use for extraction of viral RNA.

TABLE 1. Parameters defining rapid progression of SIVsmE543-3-inoculated macaques

Macaque	Survival (wk)	Plasma antigenemia[a]		Reciprocal neutralizing antibody titer[b]
		Peak	6 wk	
Rapid progressors				
Pt573	32	1.02	0.59	<32
Pt583	16	0.85	1.10	<32
Rh H445	17	17.90	29.5	<30
Slow progressors				
Rh H444	129	9.30	<0.05	6,194
Rh H460	121	1.15	<0.05	438
Rh H455	105	0.95	<0.05	3,143

[a] SIV p27 antigen in nanograms/milliliter of plasma.
[b] Reciprocol neutralizing antibody titer was assayed by cell killing assay with CEMx174 cells using SIVsmH4.

DNA extraction from frozen tissues and PCR amplification and cloning. DNA was isolated from spleen, thymus, and inguinal and mesenteric lymph nodes with standard techniques and used for PCR amplification of SIV sequences. DNA isolated from the samples listed above was used in PCR containing primer pairs to amplify the 5′ half of the genome, HD5′f (5′CCCAAGCTTTGGAAGGGATTTATTAC3′) and HD5′r (5′GCCATTCGAAGGCCTCTTCTATTG3′) and primers HD3′f (5′CAATAGAAGAGGCCTTCGAATGGC3′) and HD3′r (5′CCGCTCGAGTGCTAGGGATTTTCC3′) to amplify the 3′ half of the viral genome. The PCR products were cloned into the pCR2.1TOPO vector (Invitrogen, Carlsbad, Calif.). Ten clones from each transformation were screened, and all positive clones were sequenced.

RNA extraction from sequential plasma samples and virus amplification. Viral RNA was isolated from sequential plasma samples of Rh445 and three rhesus macaques with normal disease progression (Table 1) using the QiaAmp viral RNA kit (Qiagen, Hilden, Germany). The virion RNA from these samples was used to amplify three fragments of the envelope gene that were of interest. These fragments spanned the V1/V2/V3 region (primer pair HD1 [5′GCAATGAGATGTAATAAAACTG3′] and HD2 [5′GACCAAGGTTTCCTTCACTTCC3′]), V4/CD4 binding region (primer pair HD3 [5′CTAATGATACTAGGAAAATTAATC3′] and HD4 [5′CTCTCTTATTTCTTGAAGCACC3′]), and gp41/nef region (primer pair HD5 [5′GTTGGCTGGGATAGTGCAGCAAC3′] and HD6 [5′CCCATATGTCTCTCCACGCGCTCG3′]). Following amplification of these fragments, they were cloned directly into the pCR2.1 vector using the TOPO cloning kit (Invitrogen). Positive recombinant clones were identified using standard restriction endonuclease analysis. Ten positive clones from each time point for each fragment were sequenced and subjected to sequence analysis.

Sequencing cloned viral genomes. The 5′- and 3′-half clones of viral genomes from various tissues of the RP animals were sequenced using 18- to 20-mer oligonucleotide primers designed to be about 500 nucleotides apart and spanning the entire SIVsmE543-3 genome. Briefly, 1 μg of double-stranded plasmid DNA was mixed with 8 μl of BigDye terminator mix (PE Biosystems, Foster City, Calif.) and 3.2 pmol of each primer in a total reaction volume of 20 μl. DNA sequence reactions were performed in an automated thermal cycler (PE Biosystems). Sequencing was performed using the ABI Prism 377 automated sequencer (PE Biosystems), following the manufacturer's protocol. GeneWorks (Intelligenetics, Campbell, Calif.) sequence analysis program was used to compile alignments of both nucleotide and amino acid sequences.

Cell-cell fusion assay and immunoprecipitation analysis. Envelope sequences from 15 selected 3′ clones and the SIVsmE543-3 molecular clone were amplified from ATG to the stop codon using primers HD7 (5′ATGGGATGTCTTGGGAATCAGC3′) and HD8 (5′TCACAAGAGAGCGAGCTCAAGC3′). The PCR products were cloned into the pcDNA3.1/V5-His TOPO vector (Invitrogen) according to the manufacturer's specifications. 293 cells were transfected with 10

μg (each) of the respective envelope clones and 10 μg of the Rev expression plasmid and were labeled 24 h posttransfection by the addition of 150 to 200 uCi of [^{35}S]cysteine and [^{35}S]methionine (Amersham, Piscataway, N.J.) in Dulbecco's modified Eagle's minimum essential medium (Biofluids) lacking cysteine and methionine. The cultures were incubated overnight at 37°C and then lysed, and viral proteins were immunoprecipitated as described previously (27) using polyclonal serum from an SIVsm-infected pigtailed macaque. The immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

The cell-cell fusion assay has been described in detail previously (60). Briefly, effector QT6 cells were infected with vaccinia virus expressing T7 polymerase (vTF1.1) (1) and then transfected with a pcDNA3.1 plasmid containing the designated Env gene via the calcium phosphate transfection method. Target QT6 quail cells were transfected with plasmids expressing CD4, coreceptor, and T7 luciferase. Twenty-four hours postinfection and posttransfection, effector cells were added to the target cells and fusion was quantitated 8 h postmixing by lysing cells with 0.5% Triton X-100. An aliquot of lysate was then mixed with an equal volume of luciferase assay regent (Promega, Madison, Wis.), and luminescence was read in a luminometer (Wallac).

Nucleotide sequence accession numbers. The sequences of representative clones of the 3′ and 5′ SIV halves from each of the animals have been submitted to GenBank under accession numbers AY221508 to AY221515.

RESULTS

Virologic and pathological information has been reported previously for the three RP macaques chosen for this study, two pig-tailed macaques, Pt573 and Pt583 (21), and a rhesus macaque, Rh445 (21). Each of these animals was inoculated intravenously with the pathogenic, dual-tropic, neutralization-resistant SIVsmE543-3 molecularly cloned virus. This particular virus was derived from a short-term culture of peripheral blood mononuclear cells (PBMC) from RhE543 with CEMx174 cells (23). RhE543 exhibited opportunistic infections and florid SIV encephalitis at the time of death at 3.5 years postinoculation. The quasispecies in the brain of this animal was distinct from that observed in PBMC (8% identity), with changes in the V1, V2, and V4 regions of SU with a premature stop codon as well as additional changes in the cytoplasmic domain of the transmembrane portion of envelope.

As shown in the summary in Table 1, each of these animals fit the criteria of rapid progression. They lacked measurable SIV-specific antibody responses, as demonstrated by a lack of neutralizing antibody (shown in Table 1), exhibited persistent antigenemia, and progressed rapidly to disease in less than a year. This contrasted with the development of undetectable plasma antigen and robust neutralizing antibody responses in slower-progressing macaques, H444, H455, and H460, also inoculated with SIVsmE543-3 (21). These macaques progressed more slowly to AIDS in 128, 104, and 120 weeks, respectively. As reported previously, H445 exhibited high primary viremia (1.1×10^8 copies/ml) and increasing plasma viremia (10^9 copies/ml at death), whereas significant down-modulation of viremia was observed in the animals that progressed more slowly to AIDS (10^3 to 10^5 copies/ml by 8 weeks postchallenge) (21).

All three RP macaques were euthanized due to progressive weight loss (>10%), wasting, and persistent, unresponsive diarrhea by 16 weeks (PT 583), 17 weeks (Rh H445), and 32 weeks (Pt573) postinoculation. Pt583 also developed severe hemolytic anemia due to a malarial reactivation (*Plasmodium inui*). In each case, pathological examination revealed generalized lymphoid depletion in all lymphoid tissues as well as the gastrointestinal-associated lymphoid tissue. Severe, multifocal SIV-induced meningoencephalitis was identified in all three animals. Multinucleated giant cells expressing SIV RNA were observed in multiple tissues of all three animals by in situ hybridization (21, 23). Granulomatous, SIV-induced interstitial pneumonia was also identified for Pt573 and H445. With the possible exception of malaria, opportunistic agents were not identified on necropsy.

TABLE 2. Types of nucleotide substitutions in different genes cloned from RP macaques[a]

Clone	No. of substitutions											
	env			*tat*			*gag*			*pol*		
	Sd	Sn	ds/dn	Sd	Sn	ds/dn	Sd	Sn	ds/dn	Sd	Sn	ds/dn
445-2	1.0	13.0	0.28	0.0	1.0	N/A[b]						
445-3	4.0	13.0	1.13	2.0	0.0	N/A						
445-4	2.0	7.0	1.05	0.0	1.0	N/A						
573-1	4.0	28.0	0.52	0.0	1.0	N/A						
573-3	4.0	24.0	0.61	0.0	1.0	N/A						
573-5	2.0	28.0	0.26	0.0	1.0	N/A						
583-6	2.0	8.0	0.92	0.0	0.0	N/A						
583-8	3.0	10.0	1.10	0.0	0.0	N/A						
583-14	2.0	09.0	0.81	0.0	0.0	N/A						
573-g1							4.0	7.0	2.10	6.0	3.0	7.44
573-g2							5.0	6.0	3.06	9.0	4.0	8.39
573-g3							5.0	5.0	3.68	7.0	6.0	4.34
583-e3							4.0	7.0	2.09	3.0	11.0	1.01
583-e4							2.0	6.0	1.22	4.0	6.0	2.47
583-e5							3.0	8.0	1.38	8.0	8.0	3.73

[a] Sd, synonymous substitutions; Sn, nonsynonymous substitutions; ds/dn, ratio of synonymous to nonsynonymous substitutions.
[b] N/A, not applicable.

Total cellular DNA was extracted from the inguinal and mesenteric lymph nodes, spleen, and thymus of the three RP macaques. The 3′ portion of the SIV genome (*vpr*, *vpx*, *tat*, *rev*, *env*, and *nef*) was amplified from the tissues of each animal, and 10 representative clones from each tissue DNA sample were isolated, sequenced, and compared with the parental SIVsmE543-3 sequence. In addition, the 5′ portion of the genome (*gag*, *pol*, and *vif*) was amplified from the thymus and inguinal lymph node of Pt573 and Pt583, and 10 clones were isolated, sequenced, and compared with the parental SIVsmE543-3.

Limited and random substitutions observed in *gag*, *pol*, *nef*, *tat*, *rev*, and *vpr*. Analysis of both 5′ and 3′ clones from the RP macaques revealed that the majority of viral genes were well conserved. The average level of nucleotide substitutions relative to that for the parental strain was 0.3% in both *gag* and *pol*. A similar low number of substitutions was observed in the *vif*, *vpx*, *vpr*, *tat*, *rev*, and *nef* genes (data not shown). Interestingly, a codon-by-codon comparison of representative *gag* and *pol* genes revealed that the numbers of synonymous and nonsynonymous substitutions were nearly identical (Table 2), and there appeared to be no selection for one type of substitution. There was also no preference for nonsynonymous versus synonymous substitution in *tat* (Table 2) (50, 54). The envelope gene exhibited the most extensive variability of all the viral genes. The average level of nucleotide substitution in the envelope was calculated to be 0.7% for 37 clones analyzed. As summarized in Table 2, the majority of the nucleotide changes in the envelope region resulted in nonsynonymous amino acid substitutions (50, 54). These observations suggest that there is

TABLE 3. Range of amino acid substitutions in various proteins from RP macaque clones

Macaque	No. of substitutions								
	Gag	Pol	Vif	Vpx	Vpr	Tat	Rev	Env	Nef
RhE445	ND[a]	ND	ND	0–1	0–2	0–1	0–2	4–16	2–6
PT573	1–2	3–5	0	0–1	0–2	0–5	0–2	10–28	5–11
PT583	0–2	2–11	0–2	0–1	0–5	0–3	0–2	7–19	0–9

[a] ND, not determined.

a selection for nonsynonymous substitutions in envelope, consistent with selective pressure.

As expected from the analysis of nucleotide substitutions, significant changes from the parental virus were observed only in Env and to a lesser extent Nef (Table 3). An apparently random distribution of amino acid substitutions was observed in Gag, Pol, Vif, Vpx, Vpr, Tat, and Rev, with no substitutions being shared between more than one clone. This type of distribution of changes is shown in an alignment of representative Gag proteins from Pt573 and Pt583 in Fig. 1.

Extensive variability of envelope in tissues of RP animals. An alignment of the Env proteins cloned from mesenteric lymph nodes of RP macaques demonstrated common substitutions both within and between animals compared with the parental SIVsmE543-3 (Fig. 2 and Table 4). While many of the substitutions were observed in clones from one animal (positions 34, 55, 170, 201, 220, 355, 433, 584, 591, and 837), some were observed in all three animals at a relatively high frequency. Thus, substitutions of N158D/S, P337S/L/T, G386R, and D388N/V were observed in all three animals. The majority of clones from two of three macaques (Rh445 and Pt573) exhibited substitutions relative to the parental virus. Similar substitutions were observed in *env* clones from Pt583; however, many of the clones were wild type.

An alignment of envelope proteins from the three RP macaques revealed that the V1 region was highly conserved (Fig. 3). The classical changes in this region have been defined to be short in-frame deletions and insertions, as well as substitutions. Previous studies of SIV evolution have also revealed the acquisition of potential N-linked glycosylation sites within variable regions of the envelope (8, 12, 24, 33). In contrast, no new potential glycosylation sites were observed for *env* genes cloned from RP macaques. Indeed, one of the consistent changes was the loss of a glycosylation site in the second variable region, due to an N158D or S160N substitution (also see Table 4). A total of 20 of the 33 clones analyzed were mutated at one or both of these positions.

We observed the highest level of variability in the normally

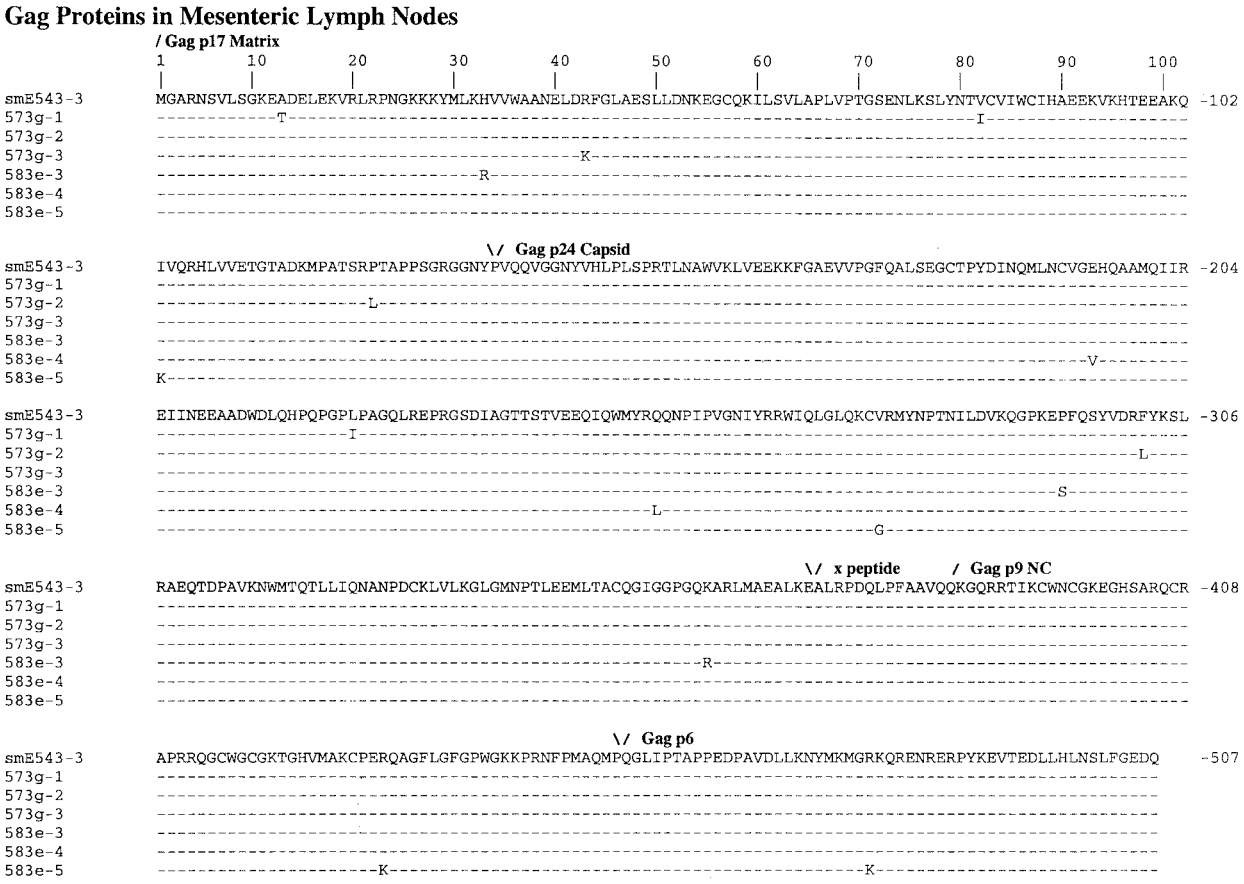

FIG. 1. Comparison of Gag proteins of various clones from the thymus and inguinal lymph node of Pt573 and Pt583, respectively. The amino acid sequence of SIVsmE543-3 is shown on the top in single-amino-acid code, and clones from Pt573 and Pt583 are shown below. The position of the various Gag proteins is indicated. Amino acid substitutions are indicated in single-amino-acid code, and identity is indicated by a dash.

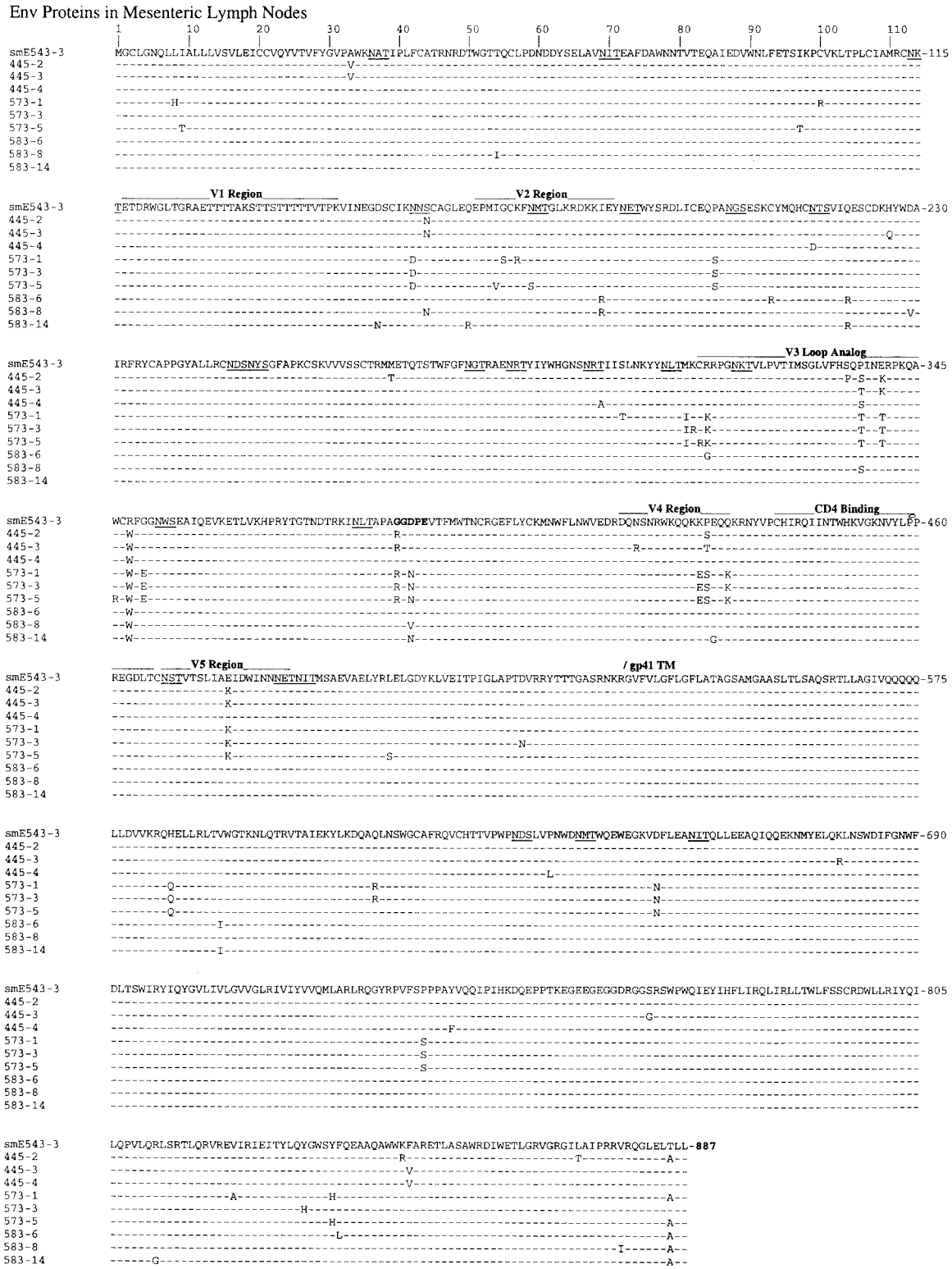

FIG. 2. Comparison of the envelope proteins from various clones from the mesenteric lymph nodes of Pt573, Pt583, and Rh445. The amino acid sequence of SIVsmE543-3 is compared with those of the other clones. Amino acid substitutions are indicated, and identity is indicated by a dash. The V1-5 regions, the conserved GDPE motif, and the transmembrane protein (gp41) are indicated above the sequence. Potential N-linked glycosylation sites are underlined.

TABLE 4. Distribution and frequency of substitutions in envelope clones from RP macaques[a]

Substitution	No. of occurrences[b]			Frequency
	Rh445 (14)	Pt573 (8)	Pt583 (11)	
A34	11	0	0	0.33
T55I0	0	0	5	0.15
N158D/S	**6**	**7**	**1**	**0.42**
S160N/G	5	0	2	0.21
158 or 160	**11**	**7**	**3**	**0.63**
I170V	0	5	0	0.15
I185T/R	2	0	4	0.18
P201S	0	6	0	0.18
Q220R	0	0	7	0.21
M312I	0	7	1	0.24
R315K/G	2	7	1	0.30
P337S/L/T	**14**	**7**	**2**	**0.69**
E340K/T	12	7	0	0.57
R348W	**13**	**7**	**11**	**0.93**
G350E	0	7	0	0.21
E355K	0	7	0	0.21
G386R	**4**	**7**	**5**	**0.48**
D388N/V	**7**	**7**	**6**	**0.60**
K429N/E	1	7	0	0.24
P430T/S	8	7	0	0.45
Q433K	0	7	0	0.21
E477K	**8**	**7**	**2**	**0.51**
D519N	5	3	0	0.24
H584Q	0	7	0	0.21
V591I	0	0	7	0.21
D635N	0	7	0	0.21
P735S	0	5	1	0.18
Y837H	0	5	0	0.15
F848V/I/S	9	0	1	0.30
T885A	**1**	**5**	**10**	**0.48**

[a] Boldface type indicates substitutions found at high frequencies in all animals.
[b] Numbers in parentheses next to animal number are the number of envelope clones evaluated.

conserved V3 loop analog as well as surrounding sequences, as shown in Fig. 3. The predominant changes in this region were P377S/T/L/H, E340K/T, and R348W. A total of 22 out of 33 clones exhibited one or all of these substitutions (Table 2). The conserved GGDPE motif in the C4 region, which had been shown to be variable in an animal infected with SIVmac239 that progressed rapidly, was also highly variable in this study. Of the 33 clones analyzed, 27 exhibited either one or both G386R and D388N/V changes. It has been postulated that this conserved motif affects the binding and affinity of the envelope glycoprotein for the CD4 receptor. The predominant changes in the V4 and V5 regions of the envelope were a P430T/S change in 15 out of 33 clones and an E477K change in 17 out of 33 clones. These substitutions were observed in only two of the animals (RhH445 and Pt573). Two associated substitutions, K429E and Q432K, were observed in the V4 region of *env* clones from Pt573. The transmembrane portion of the envelope demonstrated more animal-specific and random changes, with the exception of a T885A change, at the extreme C terminus of the envelope glycoprotein (Table 4 and Fig. 3).

As shown in Table 4, substitutions in other sites of the envelope were infrequent and were generally observed only in a small subset of clones from an animal. In contrast, Pt573 exhibited some regions of animal-specific variability relative to SIVE543-3 (I170V, P201S, E355K, K429N/E, Q433K, H584Q, D653N, P735S, and Y837H). Each of these changes was not observed in clones from the other two animals and thus appeared to be Pt573 specific. Phylogenetic analysis of the nucleotide sequence of envelope genes from various tissues of the RP animals revealed that the sequences form clusters within each animal and that there is no tissue-specific clustering of the sequences (data not shown).

To determine whether there was evidence for tissue-specific evolution of SIV in these animals, the envelope proteins from clones derived from the mesenteric lymph node (Fig. 2) were compared with those from the spleen and thymus. As shown in Fig. 3, the N158D/S, P337S/L/T, G386R, and D388N/V substitutions were observed in all three tissues.

Common substitutions in the Nef proteins. Analysis of the protein sequences of Nef of clones from the three RP macaques revealed a number of substitutions that occurred only once among the clones, similar to the pattern of changes in Gag. However, as with the Env proteins, two mutations, K83R and H169Y, were observed at a high frequency in all three of the animals (Fig. 4). In addition, substitutions were commonly observed between amino acid positions 83 and 100 immediately N-terminal to the PXXP motif. Some animal-specific changes were also observed, such as N15K/D in clones from H445 and N135S/D in clones from Pt573 and Pt583. None of the observed substitutions were in amino acid residues known to affect Nef function, with the exception of an S99P substitution that introduced a PXXPXXP motif rather than the single PXXP motif found in SIVsmE543-3. In fact, the two most consistent substitutions are common substitutions observed in the comparison of different SIVsm/SIVmac/HIV-2 Nef proteins (Los Alamos Database). Thus, both R and K residues are observed commonly at position 83, and position 169 is even more variable (T, F, C, and Y).

Unique Env substitutions appear by 8 to 12 weeks in RP macaques. Portions of the envelope were amplified from sequential plasma samples from one RP macaque (H445) and three other members of the cohort (Rh444, 455, and 460) to determine the timing of the envelope changes. These particular substitutions have not been observed in extensive prior studies of conventional progressors infected with SIVmac239, SIVmneCl8, and SIVsm62d (7, 24, 35, 55). However, we also wished to confirm that the unique constellation of *env* substitutions observed for the RP macaques were not observed for SIVsmE543-3-inoculated macaques with conventional disease progression. The conventional progressors in the present study were also inoculated with SIVsmE543-3 but developed robust SIV-specific antibody responses and survived considerably longer than any of the RP macaques (see Table 1). Variability in envelope was evaluated in plasma samples collected at 2, 16, 18, and 52 weeks for the slow progressors and at 2, 8, 12, and 16 weeks for the RP, H445. As expected, the mutations observed in tissues of H445 were also present in the plasma collected at the time of necropsy. As shown in Fig. 5, the mutations in envelope in plasma virus of H445 were first detected between 8 and 12 weeks after inoculation. The V1 region was well conserved, with the V1/V2 N158D change leading to the loss of a potential glycosylation site in the V2 region of the envelope, first appearing between 12 and 16 weeks postinoculation (Fig. 5A). This contrasted with substitutions and in-frame deletions within V1 in clones derived from plasma of the three slow progressors (the bottom of panel

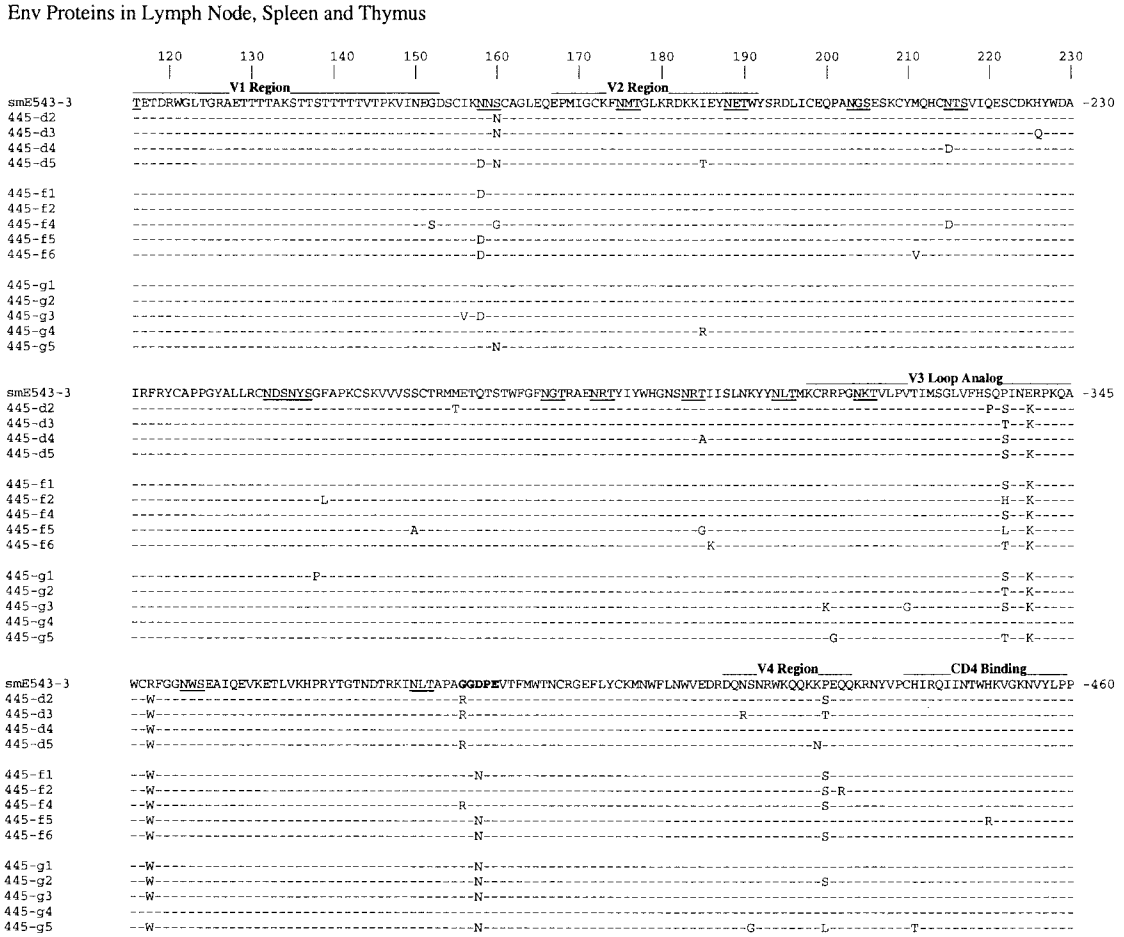

FIG. 3. Comparison of the envelope proteins from the mesenteric lymph nodes, spleen, and thymus of Pt573, Pt583, and Rh445. A partial sequence of gp120 is shown from amino acid position 116 to 460. The amino acid sequence of SIVsmE543-3 is compared with those of the other clones. Amino acid substitutions are indicated, and identity is indicated by a dash. Potential N-linked glycosylation sites are underlined. A space separates sets of sequences from different tissues.

A shows clones from 52-week samples) and conservation at position N158. The substitutions in the V3 region, P337T, E340R, and C347W, appeared between 8 and 12 weeks postinoculation in plasma samples from H445 (Fig. 5B), whereas this region remained constant in the slower-progressing macaques (bottom of panel B). Finally, the substitutions in the conserved GDPE motif in the C4 region of the envelope were also present in the circulating virus population of the RP, H445, by 12 weeks postinoculation. In contrast, this motif was highly conserved in samples from the slower progressors. Thus, the majority of substitutions in plasma virus in animals that progressed more gradually (H444, H455, and H460) were observed in the V1 region (Fig. 5A). Significant variation was not observed in other regions of envelope that were examined. Specifically, substitutions in the V3 loop analog (Fig. 4B) and GDPE motif (Fig. 5C), characteristic of all three RP macaques, were not observed even by 52 weeks postinoculation.

Envelope clones from RPs show CD4-independent use of CCR5. To evaluate the functional relevance of the substitutions observed in Env proteins from RP macaques, 15 3′ clones were chosen based on their representation of various envelope substitutions. The envelope genes from these clones were amplified and subsequently cloned into the pcDNA3.1 vector with the SIVsmE543-3 envelope as a positive control. Although all clones expressed Env, processing from gp160 to gp120 was observed with only six of the clones (Fig. 6A). Five of these clones were competent for fusion in a cell-cell fusion assay (clones 1, 2, 5, 6, and 9). A discrepancy between concurrence between fusion and processing was observed for clones 14 and 15. Since the fusion assays were repeated multiple times, we presume that clone 14 was also capable of processing and the lack of processing was due to an inadvertent switch between clones 14 and 15 in the radioimmunoprecipitation analysis. As shown in Fig. 6B, each of these clones utilized CCR5 as efficiently in the absence of CD4 as in its presence. This contrasted with largely CD4-dependent usage of CCR5 by parental SIVsmE543-3 Env. The RP Env clones also demonstrated a diminished ability to use other coreceptors, such as CXCR6 (Strl33), Gpr15, CXCR4, APJ, and CCRX. Examination of the sequence of the six functional clones in Fig. 7 revealed that all had substitutions within the GDPE motif, three had the G386R substitution, one had D388N, and two contained both substitutions. Four of the clones had substitutions downstream of V1 that resulted in the loss of an N-linked glycosylation site,

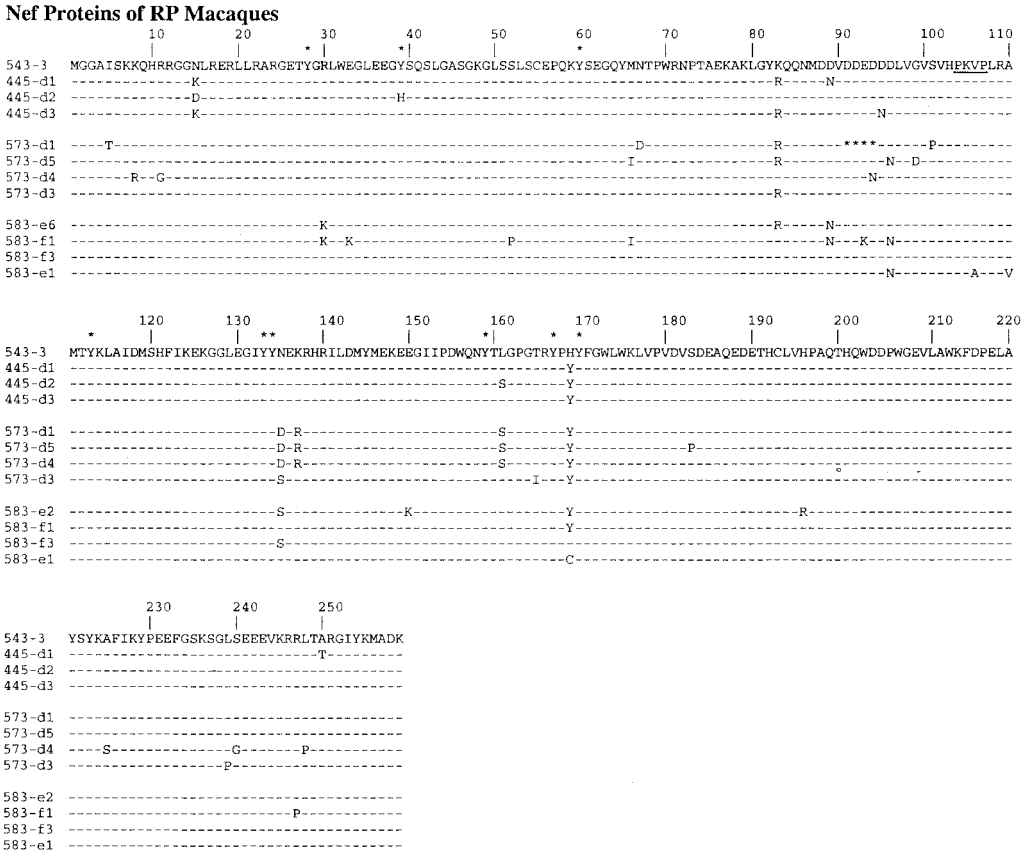

FIG. 4. Comparison of the Nef proteins from various clones from the mesenteric lymph nodes of Pt573, Pt583, and Rh445. The amino acid sequence of SIVsmE543-3 Nef is compared with those of RP clones. Amino acid substitutions are indicated, and identity is indicated by a dash. Tyrosine residues are indicated by an asterisk, and the PXXP motif is underlined.

and five of the clones had substitutions within the V3 loop analog. Thus, each of the clones exhibited multiple RP-specific Env mutations that might contribute to CD4 independence.

DISCUSSION

In the present study, we demonstrated a unique pattern of sequence evolution in animals that developed rapid disease in the absence of measurable antibody responses. The location of substitutions in the envelope gene differed from results in previous studies on animals with slower disease progression (3, 4, 6–8, 12, 26, 35, 61). Instead of substitutions within the previously defined variable regions, V1, V2, V4, and V5, consistent substitutions were concentrated within the V2 to V3 domains of envelope in all three animals examined. The common substitutions observed included the following: (i) N158D or S160N/G, resulting in the loss of a glycosylation site in V1/V2; (ii) substitutions in the V3 loop analog, P337S/L/T, E340K/T, and R348W; and (iii) G386R and/or D388N/V in the highly conserved GDPE motif in C4. Examination of sequences in the plasma of one of the RP macaques revealed that the characteristic substitutions appeared between 8 and 12 weeks postinoculation and were maintained until death in the plasma and in tissues. Therefore, they develop after the loss of cellular and humoral immune responses.

Previous studies of in vivo SIV evolution have focused mostly on macaques that progressed to AIDS in the face of persistent cellular and humoral immune responses (3, 4, 6–8, 12, 26, 35, 61). Four concentrated regions of sequence variability in gp120 were observed (7, 29). These have been defined by analogy to the HIV-1 envelope as the V1, V2, V4, and V5 regions. The hallmark of sequence evolution in SIV infection is amino acid substitutions, insertions, and deletions, primarily in the V1 region of the envelope, substitutions in the V4 and V5 regions, and the acquisition of potential N-linked glycosylation sites through the SU portion of the envelope (12). These additional glycosylation sites presumably evolve as a method of immune escape from neutralizing antibodies. The V3 region (or V3 loop), which is the most variable region of HIV-1 gp120, is a critical determinant of lymphocyte/macrophage tropism and coreceptor usage (5) as well as a type-specific linear neutralization determinant. However, the analogous cysteine loop of SIV (termed the V3 loop analog) is generally highly conserved. Nevertheless, spontaneous or genetically engineered substitutions in the V3 loop analog of SIV alter the tropism of SIV, suggesting that this region interacts with the viral coreceptors (26, 36).

The virus in HIV-infected humans and SIV-infected macaques also evolves in terms of biologic properties. In HIV infection, progression to AIDS is frequently associated with a

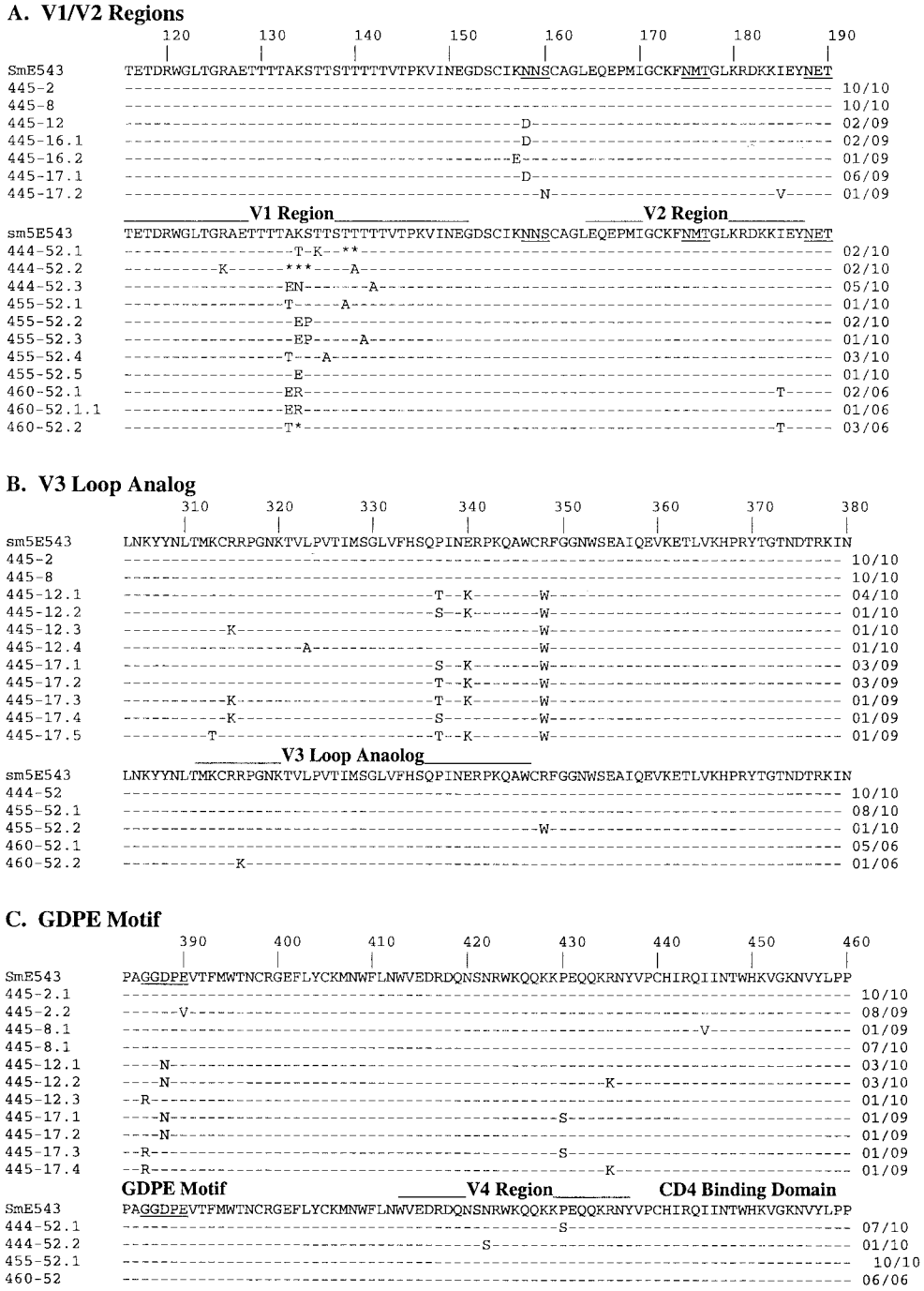

FIG. 5. Comparison of the V1 and V2 regions (A), V3 loop analog (B), and GDPE motif and V4 region (C) in sequential plasma samples from Rh445 (top) and Rh444, Rh455, and Rh460 (normal progressors). The amino acid sequences derived from plasma viral RNA are compared to that of SIVsmE543-3. Amino acid substitutions are indicated, identity is indicated by a dash, and an asterisk indicates in-frame deletions. Sequential samples from 2, 8, and 17 weeks postinfection of the RP, H445, are at the top portion of each panel. Env clones from 52 weeks postinoculation from the progressors, 444, 455, and 460, are in the bottom portion of each panel. The time of plasma sample collection is indicated in terms of weeks postinoculation. The number of clones showing the indicated sequence is shown. The V1 to V5 regions and the GDPE motif are indicated above the sequence.

switch in coreceptor usage from CCR5 to CXCR4 (13). However, a coreceptor switch is clearly not required for the development of AIDS, since many patients develop AIDS in its absence (11). Interestingly, changes in coreceptor usage have not been observed during SIV progression to AIDS, in keeping with the highly conserved nature of the V3 loop analog of SIV. However, progression is frequently associated with increased replicative properties of the virus in primary cells, such as PBMC or macrophages, and increased resistance to neutralizing antibodies (32–35). Studies with the moderately pathogenic

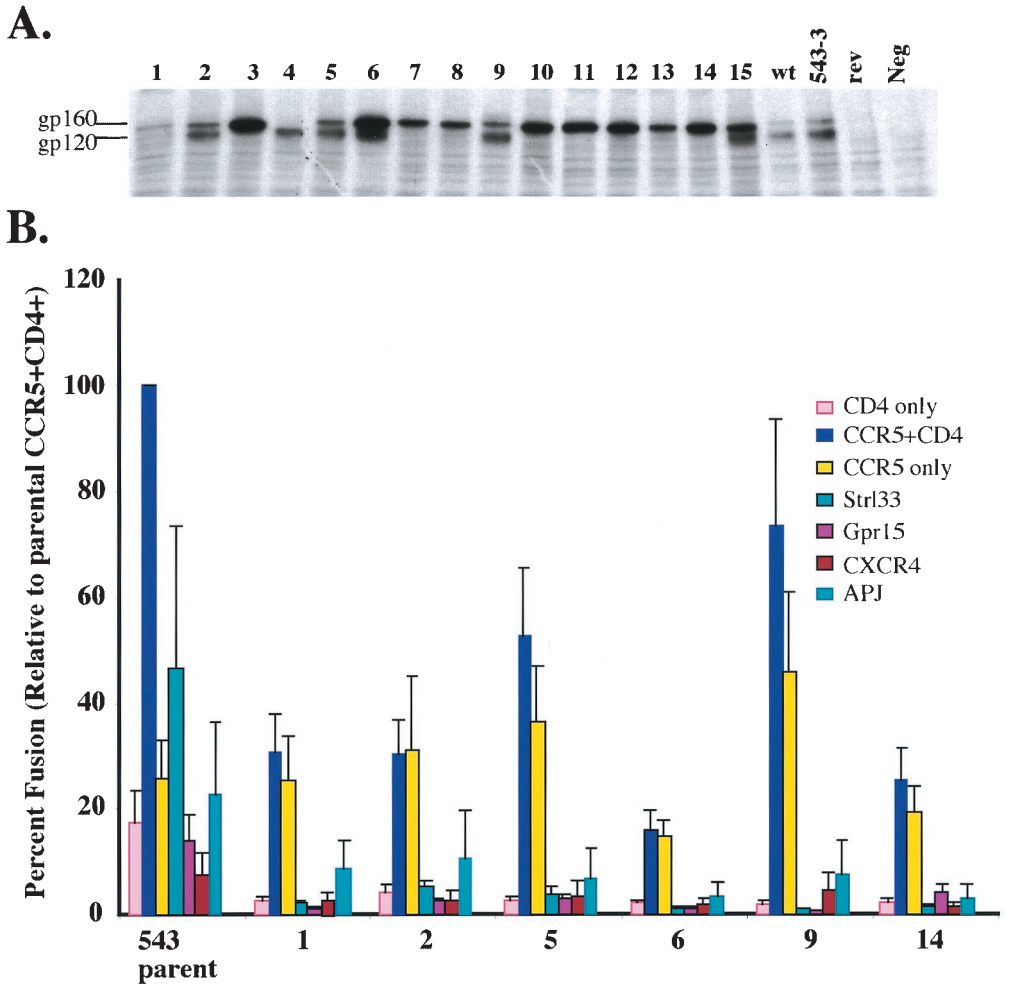

FIG. 6. Envelope and coreceptor usage of representative envelope clones from RP macaques. Panel A shows radioimmunoprecipitation analysis of envelope proteins following cotransfection of 15 representative envelope clones with a *rev* expression plasmid into 293 cells. All clones express gp160, although the expression level varies. Processing to gp120 is observed in only six of the clones (1, 2, 5, 6, 9, and 15). Panel B shows the patterns of CD4 and coreceptor usage for the clones represented in panel A, expressed as a percentage of the fusion observed for CCR5 plus CD4 for parental SIVsmE543-3 envelope.

SIVmneCl8 have demonstrated that the virus isolated from the later stages of disease is more virulent when used as an inoculum in naive macaques, consistent with evolution of the pathogenic potential of the virus in vivo (62). This increased virulence is associated with the acquisition of glycosylation sites in gp120 as well as changes in other genes of the virus and increased resistance to neutralization and increased replicative capacity (33). Similar acquisition in glycosylation sites has been observed during in vivo evolution of antigenic variants of SIVmac239 (6, 7, 35).

A few studies of in vivo SIV evolution have included some RP macaques inoculated with either uncloned SIVsmE660 or SIVmac239 (8, 27, 37). A common feature of envelope genes cloned from RP macaques in previous studies were substitutions within the V3 loop analog and in the conserved GDPE motif (8, 27, 37). Substitutions in the conserved GDPE motif as well as in the V3 domain were also observed in SIVmac239-infected animals with encephalitis. The studies of evolution of macrophage-tropic variants in SIVmac239-infected macaques with SIV-induced encephalitis have incidentally included RP macaques. This is not surprising, since RP macaques, including the three in the present study, frequently exhibit SIV-induced encephalitis. Likewise, the development of SIV-induced encephalitis is associated with rapid disease progression in the SIVmac model (70, 72). Two macrophage-tropic variants, SIVmac316 (47) and SIVmac17E-Br, have been defined from studies of SIVmac239-infected macaques with neuro-AIDS (4). Both of these isolates are macrophage tropic and neutralization sensitive and use CCR5 independently of CD4 (17, 58). SIVmac17E-Br shares some of the envelope sequence changes characteristic of SIVmac316, in particular the V67 M substitution and the G383R substitution in the GDPE motif. However, SIVmac17E-Br has a substitution at proline 334 in the V3 loop, while this amino acid is wild type in SIVmac316. Similar to brain envelopes of RhE543, SIVmac17E-Br and SIVmac316 encode a prematurely truncated transmembrane glycoprotein.

In the present study of SIVsmE543-3 evolution in lymphoid tissues of RP macaques, we observed similar substitutions as observed in the SIVmac-infected macaques with encephalitis. Specifically, we observed substitutions at proline 337 (334 in

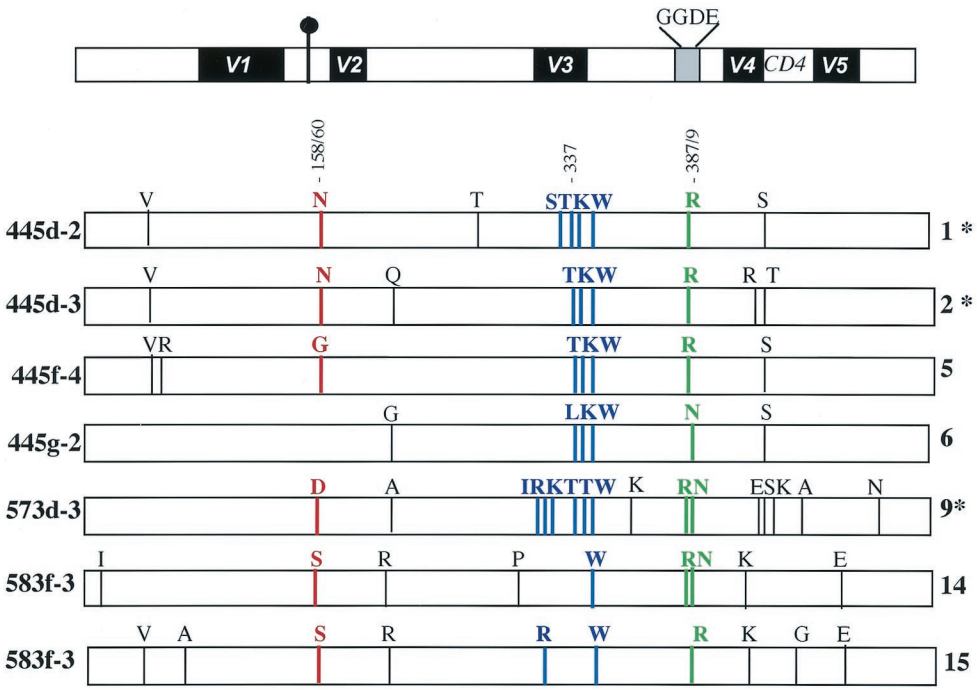

FIG. 7. Schematic representation of amino acid substitutions relative to the parental SIVsmE543-3 in the six functional RP envelope clones. The source of each clone is detailed on the left. The common substitutions characterized in RP macaques are highlighted in color: the loss of glycosylation in V1/V2 in red, the changes in the V3 loop in blue, and the changes in the GDPE motif in green. The various variable and functional regions are detailed at the top. The complete envelope sequences of three of these clones (1-1, 2-6, and 9) are shown in detail in Fig. 2.

SIVmac) of the V3 loop analog and the changes in the GDPE motif. In addition, we observed substitutions in the V1/V2 region that resulted in the loss of a potential N-linked glycosylation site. Although the biologic effect of loss of this glycosylation site in SIV envelope has not been defined, loss of glycosylation sites in the V2 region of HIV-1 has been associated with CD4-independent replication of CCR5-using primary isolates of human immunodeficiency virus (38). The V1/V2 region of HIV-1 is thought to represent a large surface loop that covers the V3 loop, which is involved in coreceptor interaction (38, 71). Upon binding CD4, the V1/V2 loop moves, exposing V3 for interaction with its coreceptor. Therefore, loss of glycosylation in this region could potentially expose the coreceptor binding site or allow greater fluidity of movement, allowing CD4-independent infection. The loss of glycosylation in the V1/V2 region of SIVmacE543-3, in tissue clones from Mm316-85, and in SIVmac17E-Fr suggests that this particular substitution is likely to affect coreceptor binding and may be responsible in part for a CD4-independent phenotype. Loss of this glycosylation site has also been associated with increased neutralization sensitivity of HIV-1 (59). In support of a role for loss of V1/V2 glycosylation in the CD4-independent phenotype of our SIVsm clones, SIVmac239 envelopes with deletions of glycosylation sites in V1/V2 have also gained CD4-independent function (58). Previous studies of SIVmac239 derivatives that are CD4 independent (316, 17E-Fr, and 1A11) have demonstrated an association between CD4 independence, macrophage tropism, neutralization sensitivity, and attenuation in vivo (43, 58).

Convergent evolution of envelope of SIV is unusual and suggests that viruses expressing such envelope sequences possess a selective advantage in the cellular and immunologic milieu that occurs in RP macaques that lack measurable humoral immune pressure. Studies of the cellular immune responses of other RP macaques revealed transient cytotoxic-T-cell responses that had waned by 3 weeks postchallenge (65). Thus, we believe that such RP macaques lack both humoral and cellular immune responses and selective pressure on virus evolution. The lack of an SIV-specific immune response leads us to conclude that evolution of SIV variants in RP animals is driven by selective pressures other than immune responses. Previous studies of antigenic variants of SIV that have escaped neutralization suggest that acquisition of glycosylation sites in V4 as well as insertion/deletion changes in V1 are responsible for escape from neutralization (6, 7, 35). Thus, it is no surprise that the V1 region remains fairly conserved in RP macaques and that the only changes in glycosylation are the loss of a site in the V1/V2 region. The majority of SIV-infected cells in lymphoid tissues of these animals appeared to be cells of macrophage origin, principally multinucleated giant cells (23). The most obvious selective pressure that the virus could face in a host without an active immune response would be the need to infect and replicate in different cell types. Interestingly, the primary cell type that harbors SIV in RP macaques appears to be macrophages rather than $CD4^+$ T cells (unpublished data), suggesting that the virus has evolved the ability to replicate more efficiently in macrophages. A similar switch to growth in macrophages occurs in macaques infected with pathogenic CXCR4-using SHIV viruses, such as DH12R (28). In this situation, the switch in tropism is presumed to be due to the

selective and rather complete depletion of $CD4^+$ T cells in such animals. Although SIV-infected RP macaques maintain moderate numbers of $CD4^+$ T cells, it is possible that the cells in such animals are dysfunctional and therefore are not a good target for SIV replication. We hypothesize that the $CD4^+$ T cells of SIV-infected RP macaques that fail to maintain SIV-specific immune responses are incapable of proliferating and therefore are ineffective cell substrates for SIV replication. This would force the virus to adapt in order to more efficiently replicate in macrophages and potentially also to mediate CD4-independent entry, since macrophages express low levels of CD4 (48).

Representative clones from SIVmacE543-3-infected RP macaques utilized CCR5 independently of the presence of CD4. These clones all possessed multiple signature substitutions characteristic of RP envelopes. Four clones had the V1/V2 glycosylation site loss, as well as substitutions in V3 and the GDPE motif. One clone had the V3 loop and GDPE substitutions, and one had only substitutions in the GDPE motif. Yet all were phenotypically similar in fusion assays. Possibly these three mutations are functionally redundant, all producing a similar phenotype. Detailed dissection of the contribution of each of these three mutations to CD4 independence and other properties conferred by these envelopes will be necessary. A recent study of envelope clones from RP macaques inoculated with SIVmac239 also demonstrated the predominance of substitutions in the GDPE motif and a similar association with CD4 independence (63). However, the function of the other two signature mutations is not clear. Substitutions in the V3 loop analog could influence coreceptor interactions. However, all of the envelopes in our study with V3 loop substitutions maintained usage of CCR5 and did not exhibit broadening to include other coreceptors, such as CXCR4. Indeed, these envelopes appeared to utilize other coreceptors with less efficiency than the parental envelope. The loss of glycosylation in V1/V2 could potentially lead to exposure of the V3 loop and thus greater sensitivity to neutralization as well as CD4 independence.

Collectively, the data presented here indicate that the pattern of SIV evolution in the absence of host immune pressure is unique and differs from the pattern observed in animals which progress normally to AIDS in the face of persistent immune responses. Previous studies of macrophage-tropic variants of SIVmac239 have suggested that this unique pattern of evolution is associated with the development of SIV-induced encephalitis (64). However, the observation of these specific variants in lymphoid tissues in the present study, as well as the universal presence of giant cells in multiple tissues of such animals, suggests the simultaneous, concurrent evolution of these unique SIV envelope variants in multiple tissue compartments. We therefore believe that this specific pattern of evolution results primarily from the lack of immune responses in these animals and the subsequent development of encephalitis is driven by the specific pattern of evolution of the virus. Finally, it is not clear whether the unique pattern of evolution of SIV in RP macaques contributes to the rapid disease phenotype in these animals or is simply a consequence of viral evolution in the absence of antiviral immunity.

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