JOURNAL OF VIROLOGY, May 1994, p. 3129–3137
0022-538X/94/$04.00+0

Vol. 68, No. 5

# Extensive Envelope Heterogeneity of Simian Immunodeficiency Virus in Tissues from Infected Macaques

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Received 1 September 1993/Accepted 7 February 1994

**The extent of virus genetic variation within tissues and peripheral blood mononuclear cells (PBMC) from two simian immunodeficiency virus (SIV)-infected macaques was analyzed. The products of PCR amplification of two regions, region 1 (SIV V1 region) and region 2 (region corresponding to the human immunodeficiency virus V3 cysteine loop and part of the C3 region immediately downstream), of the SIV envelope were examined for single-stranded conformation polymorphism followed by sequence analysis of selected clones. The V1 region of the SIV envelope of viruses present within lymphoid tissues displayed extensive heterogeneity, while viral populations within the PBMC and brain appeared to be less variable. Region 2 heterogeneity in both animals was generally confined to three residues in a tissue-specific manner. In addition, virus from the brains of both animals appeared to be distinct compared with viruses present in other tissues and PBMC of the same animal, both in the pattern of PCR–single-stranded conformation polymorphism SCP and in the sequence of region 2. These studies revealed that the tissues of SIV-infected macaques were a reservoir for viral variants distinct from those seen in PBMC.**

The genetic and biologic diversity of human immunodeficiency virus (HIV) is thought to be one of the major determinants for enhanced survival of the virus within an infected individual; this diversity is characterized by the presence of a virus quasispecies within infected peripheral blood mononuclear cells (PBMC) (1, 15, 18, 36). HIV exists as a quasispecies in vivo most likely as a result of the high mutation frequency and lack of an error correction mechanism of reverse transcriptase (6, 16, 17). As a consequence, the existence of this large population pool allows for the selection of variants that have the potential to escape from the host immune response or grow in different cell types and environments. The most extensive variation has been observed within the envelope surface glycoprotein (17), where several epitopes seem to be key targets for the immune system (7, 35, 50). In fact, the major neutralization domain of the HIV envelope is the hypervariable V3 loop (35, 50). Neutralization escape mutants have been observed following infection with HIV (1, 39, 48, 49). In addition to the potential production of immune escape variants, HIV envelope heterogeneity may alter cellular tropism. For example, variation of only a few amino acids within the V3 loop can affect the ability of HIV to infect macrophages in culture (26, 40, 45, 46, 52). The majority of these studies have examined HIV envelope variation within PBMC of infected individuals. However, lymphoid and other tissues such as the brain are major reservoirs of HIV (12, 14, 43, 44). The brain appears to harbor viral variants of distinct genetic and biologic properties (14, 43). However, little is known about the type and extent of variation in lymphoid tissues, although analysis of V3 sequences from splenic and brain viruses suggests that the separate evolution of tissue-specific variants occurs (14).

Simian immunodeficiency virus (SIV) infection of macaques is a useful and relevant model for understanding the pathogenesis and genetic variation of HIV infections (2, 11, 25, 33). SIV, like HIV, infects both macrophages and CD4 lymphocytes and induces a similar immunodeficiency syndrome in experimentally infected monkeys. Importantly, SIV exists as a quasispecies in the infected animal (29) and also evolves during the course of infection, generating neutralization escape variants (8). Also as for HIV, while there has been extensive research of SIV variation in PBMC from infected macaques (3, 9, 29, 41, 42), little is known about specific viral variants found in tissues. An apparently high degree of envelope heterogeneity has been observed during analysis of DNA derived directly from the spleen of an SIV-infected pigtail macaque (*Macaca nemestrina*) (25). In contrast to the extensive heterogeneity observed in this study of the spleen, variation in lymph nodes from another study of infected macaques generally paralleled that observed from PBMC at less than 1 year postinfection (41, 42). As in humans infected with HIV, macrophages harbor SIV in infected tissues such as the brain (5, 32, 38). Viral DNA from several tissues (including the brain and gut) of rhesus macaques infected with $SIV_{mac239}$ had significant amounts of variation in at least two regions of the envelope gene (32). However, the extent of virus variation between tissues in an infected macaque has not been clearly established. Although SIV is generally considered an ideal model for human AIDS, there are some important differences between SIV and HIV. First, while there are several analogous hypervariable regions in the SIV envelope (V1, V2, V4, and V5), none specifically corresponds to a neutralization epitope (8, 28, 31). Second, unlike the HIV V3 loop, the analogous SIV V3 region is much more conserved in sequence (9, 29, 41) and has not been shown to correspond to a specific linear neutralization epitope (8, 28, 31). However studies in this laboratory demonstrate that this region is involved in cellular tropism of $SIV_{sm}$ (24a).

The goals of this study were to investigate SIV envelope heterogeneity in lymphoid and nonlymphoid tissues, compared with the variation in the peripheral blood, and to determine whether the variation observed was tissue specific. In addition, the extent and type of variation may suggest whether such pressures as cellular tropism or immune selection play a role in

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the genetic diversity of the SIV envelope in tissues other than PBMC. In this study, SIV envelope variation was analyzed directly from specific tissues by using PCR followed by both single-stranded conformation polymorphism (SSCP) and sequence analysis. Comparisons focused on the most variable region (V1) of the SIV envelope and region 2 (region corresponding to the HIV V3 loop and part of the conserved region immediately downstream).

## MATERIALS AND METHODS

**Virus and Animals.** Two macaques were inoculated with related $SIV_{sm}$ strains. Rhesus macaque (*Macaca mulatta*) E543 was inoculated with $SIV_{sm/F236}$, which has been described previously (25). Briefly, SIV was isolated from rhesus macaque F236 by cocultivation with H9 cells and filtered culture supernatant used to infect RhE543. Pigtail macaque (*M. nemestrina*) Pt56 was infected with cell-free virus isolated from CEMX174 cells incubated with PBMC from rhesus macaque 660 (Rh660) (25). This donor animal, Rh660, was infected previously with lymph node (LN) cells from RhE543. Related sequence information about these virus inocula is presented below.

**Disease progression in monkeys.** Although both animals had SIV-induced meningoencephalitis (47) with multiple foci of syncytial cells, they differed notably in clinical course of disease. RhE543 was persistently viremic and had a strong antibody response against all major SIV proteins throughout the course of infection. High titers of neutralizing antibodies were present throughout the course of infection (unpublished data). The monkey was sacrificed 42 months after infection principally as a result of SIV-induced meningoencephalitis. In contrast, while Pt56 was also persistently viremic, this monkey developed only a transient antibody reactivity against the SIV envelope surface protein, as detected by Western immunoblotting (25), and never developed antibodies to the SIV Gag protein. Pt56 serum was not tested for the presence of neutralizing antibodies. Pt56 was sacrificed 14 months after inoculation primarily because of opportunistic infections resulting from profound immunosuppression (25). In addition to meningoencephalitis, pathological findings at necropsy of Pt56 included interstitial histiocytic pneumonia and disseminated mycobacterium infection. Various tissues and PBMC were obtained at necropsy from the two macaques, snap frozen, and stored at −70°C as described previously (25).

**DNA isolation.** DNA was isolated from PBMC, brain, spleen, axillary LN, mesenteric LN, and ileum from RhE543, using standard techniques (23) in a separate room used only for PCR to limit possible SIV plasmid contamination. DNA was isolated previously from various tissues from Pt56: PBMC, brain, spleen, mesenteric LN, lung, and ileum (25).

**PCR amplification and cloning.** DNA (100 ng) from samples prepared as described above was used in nested PCRs containing primers env 1F/env 1R (outer) and env 2F/env 2R (inner) to amplify the *env* gene as described previously (29). The PCR conditions were 30 cycles of 94°C for 1 min, 55°C for 1.5 minut, and 72°C for 3 min. Ten microliters from the first-round reaction was transferred to new tubes with fresh reagents for second-round reactions. Perkin-Elmer Cetus reagents and equipment were used in all PCRs as specified by the manufacturer. A fraction of the product was analyzed on a 0.9% agarose gel, and the remainder was digested with *Csp*45I and *Sph*I and cloned into a pGEM-7Zf (+) vector (Promega). Twenty clones from each tissue or from PBMC were identified by colony hybridization with a radiolabeled probe representing the entire *env* gene and were used in PCR-SSCP and sequencing reactions.

**PCR-SSCP.** Two well-characterized variable regions in the *env* gene, V1 and region 2 (corresponding to amino acids 320 to 399 of the $SIV_{smH4}$ envelope), as well as a highly conserved region of the integrase (Int) domain of *pol*, were analyzed by PCR-SSCP. PCR-SSCP involves incorporation of radiolabeled $^{32}P$ into PCR products, followed by analysis of the denatured, single-stranded products on a neutral polyacrylamide gel (19, 20, 27). Two rounds of amplification of 25 cycles each were required for sufficient signal for PCR-SSCP analysis of tissue DNA. However, only one round of amplification of 25 cycles was required for the detection of PCR-SSCP products when plasmid clones were used. The PCR conditions for the first round of amplification of the entire gp120 gene of *env* were denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 3 min. For the first round of the integrase region, as well as all second-round reactions, the times of the PCR were modified to 30 s for each step, while the temperatures were not altered. Sequences of the primers used in the PCRs are as follows:

| | | | | |
|---|---|---|---|---|
| Env | R1 | | F | TGGGATGTCTTGGGAATCAGCTGCTTA (6588) |
| | | | R | CTTTTCTTGCTGAATTTGTGCTTCTTC (8596) |
| | R2 | V1 | F | CTCACCCCACTATGTATAGCAATGAGA (6896) |
| | | | R | AATTACAACCTATCATGGGCTCCTGTT (7098) |
| | | Reg2 | F | GATCAAGCTTAATAAGTATTATAATCTAAC (7493) |
| | | | R | GATCCTCGAGTCTACAATTTGTCCACAT (7774) |
| Int | R1 | | F | CTATTACAGAGAAGGCAGAGACCAGCTGTG (5191) |
| | | | R | GCTATGCCACCTCTCTAGCCTCTCCGGTATCCT (5399) |
| | R2 | | F | ACTAAGCTTTGTCCAAAGGGGAAGGAGCAGTCA (5246) |
| | | | R | ACTCTCGCGACTATCCAATTCTTTTCCTCCTCCATA (5354) |

Actual genome coordinates from $SIV_{psmH4}$ of the underlined nucleotide are given in parentheses. The extra 5′ nucleotides in some of the primers were introduced for restriction enzyme cloning. R1, R2, F, R, and Reg2 denote round 1, round 2, forward, reverse, and region 2, respectively. In addition to the recommended concentrations of deoxynucleoside triphosphates, 0.5 mCi of [α-$^{32}P$]dCTP was used in each second-round reaction (or only round for plasmid clones) to radiolabel the PCR products. Three microliters of a 25-μl reaction was diluted 1:1 with Sequenase stop buffer (U.S. Biochemical), denatured by boiling for 5 min, and loaded on a 10% glycerol–6% hydrolink gel (AT Biochem, Malvern, Pa.) in 0.6× Tris-borate-EDTA running buffer. The gels were run at constant power (30 W) for 5 h at room temperature, dried, and exposed overnight to X-ray film at −70°C. Plasmid clones were scored as identical in the target region when identical migration patterns were observed on the autoradiograph (comigration of both double-stranded and single-stranded bands). PCR-SSCP reactions were performed in duplicate on all tissues and clones.

**Sequence determination and analysis.** The virus stocks used for inoculation of the two study animals were partially sequenced previously (24, 25, 29). Briefly, smH4i and smH3, full-length clones derived from the F236 chronically infected H9 cell line (RhE543 virus inoculum), differ in only five amino acids (nine nucleotide differences) in the envelope gene (24, 29). There were no differences in either the V1 region or region 2 of smH4i and smH3. Sequences of eight additional clones of the V1 region of virus DNA derived from the same cell line failed to indicate significant heterogeneity (unpublished data). Little or no variation (two or fewer amino acids) was observed in the V1 region from 10 clones derived from CEM cells infected with the 660 virus (Pt56 virus inoculum) (25) or from three clones in region 2 (unpublished data).

The multiple sequence alignment program Clustal V was used for comparisons of sequences derived from several clones

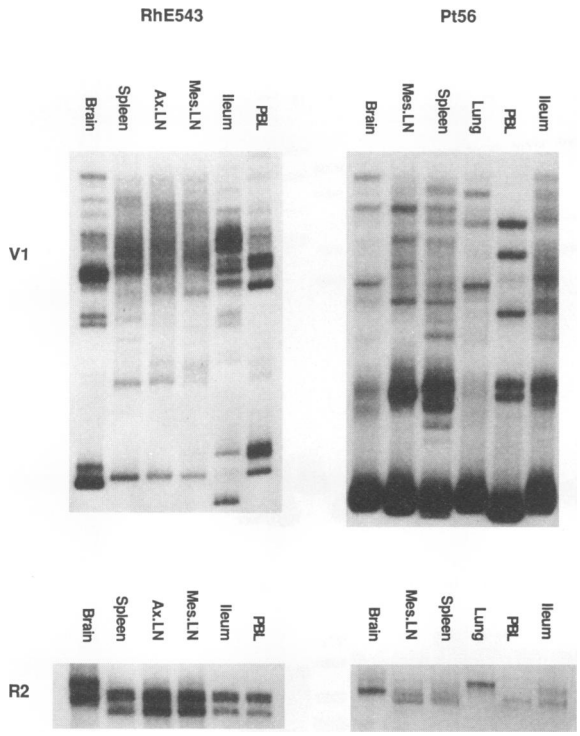


FIG. 1. PCR-SSCP analysis of DNA from tissues and PBMC of RhE543 and Pt56. Two regions of the SIV envelope, V1 and region 2 (R2), from the indicated tissues of the two infected macaques were examined for variability. The bands in the upper two-thirds of each V1 autoradiogram show specific single-stranded conformations of the double-stranded amplified product (strong bands at bottom of each V1 gel). The double-stranded products of region 2 were omitted in these photographs because there was no detectable variation in the sizes observed on the gels. Ax.LN, axillary LN; mes.LN, mesenteric LN.

from each animal (21, 22). Alignments of the predicted amino acids of individual clones of various regions of the *env* gene were also performed by using Clustal V.

**Nucleotide sequence accession numbers.** All sequences listed in Fig. 4 to 6 have been submitted to GenBank and assigned accession numbers U05049 to U05191.

## RESULTS

**Direct PCR-SSCP analysis of SIV heterogeneity in tissues.** The general pattern of virus variation within tissues and PBMC of RhE543 and Pt56 was examined; PCR-amplified products of the entire gp120 gene were subjected to a second round of PCR (PCR-SSCP), using nested primers spanning either the V1 region or region 2 of the SIV *env* gene. Control PCR-SSCP reactions of the integrase region were performed to demonstrate the fidelity of the PCR. As expected, no detectable variation within the integrase region was observed (data not shown). In contrast, considerable heterogeneity was observed in the V1 region of viruses within most tissues of either animal (Fig. 1), as evidenced by the multitude of single-stranded products in the upper two-thirds of the spleen and LN lanes in both animals. Double-stranded products also varied considerably in size, especially in the samples from RhE543, suggesting that insertions and deletions occurred within the V1 region. While there was extensive heterogeneity of virus in the lymphoid tissues, limited heterogeneity was observed within the brain of RhE543 and the brain, lung, and PBMC of Pt56; one or two species predominated, with the presence of limited numbers of single-stranded products, generally fewer than five major bands (greater than 50% of the products).

TABLE 1. Analysis of nucleotide changes in tissue-derived clones

| Monkey | Region | % Transitions[a] | | | | % Nonsynonymous substitutions[b] |
|---|---|---|---|---|---|---|
| | | G→A | A→G | C→T | T→C | |
| RhE543 | V1 | 29 | 12 | 1 | 0 | 96 |
| | R2 | 20 | 27 | 1 | 33 | 76 |
| Pt56 | V1 | 28 | 22 | 25 | 0 | 95 |
| | R2 | 32 | 16 | 28 | 0.5 | 79 |

[a] Derived from the numbers of each type of transition in the region/total number of transitions for each tissue from individual monkeys.
[b] See footnote *a* for derivation of frequencies. A nonsynonomous substitution results in an amino acid change.

In contrast to the extensive variability in the V1 region, considerably less variation of the single- or double-stranded PCR-SSCP products was observed within region 2. However, distinct migration patterns of PCR-SSCP products were detected from both brain samples and also the lung of Pt56, suggesting that these tissues harbor unique genotypes.

**PCR-SSCP analysis of *env* clones.** Twenty full-length envelope genes cloned from each tissue of RhE543 and Pt56 were analyzed by PCR-SSCP to determine the frequencies of individual V1 region and region 2 variants. The extent of heterogeneity of all clones was analyzed on individual gels, and PCR-SSCP reactions of the V1 region of clones from three RhE543 tissues (brain, mesenteric LN, and PBMC) are illustrated in Fig. 2. There were clear differences in the SSCP patterns between the tissues. For instance, while clones derived from the brain (Fig. 2A) were relatively homogeneous (identical migration patterns of clones 2, 4, 6, 8, 9, 10, 14 to 17, 19, and 20), a wide array of virus variants were observed in lymphoid tissues such as the mesenteric LN (Fig. 2B; one group of four [clones 2, 12, 13, and 19], one group of three [clones 1, 5, and 17], and two groups of two [clones 10 and 15 and clones 4 and 9]; all of the other clones were unique). In general, the variability seen among envelope clones reflected the heterogeneity observed in the PCR-SSCP reactions performed directly with tissue-specific genomic DNA. For instance, the virus clones from the brain of RhE543 and the PBMC of Pt56 had the least amount of variation in V1 (80 and 90%, respectively, consisted of two or one major species) compared with the virus clones from the LN or spleen from either animal, in which no variants represented greater than 25% of the species (Fig. 3). The virus cloned from the brain, lung, and PBMC of either animal was generally less complex than that cloned from the LN and spleen. However, clones amplified from the PBMC of RhE543 and the lung of Pt56 (data not shown) displayed a larger degree of heterogeneity than that observed during analyses of other nonlymphoid tissues and the PBMC of Pt56. Interestingly, the heterogeneity of the virus from all RhE543 tissues and PBMC was less than that from Pt56 in region 2 (data not shown).

**Sequence heterogeneity of the V1 region and region 2.** The V1 region and region 2 from several of the PCR-derived envelope clones were sequenced to determine (i) the accuracy of PCR-SSCP analysis in predicting nucleic acid heterogeneity, (ii) the types of nucleic acid changes observed in different tissues, and (iii) whether any of these changes could be explained by selective pressures such as immune selection (3, 8, 42) or presumed changes in cell tropism (37, 38).

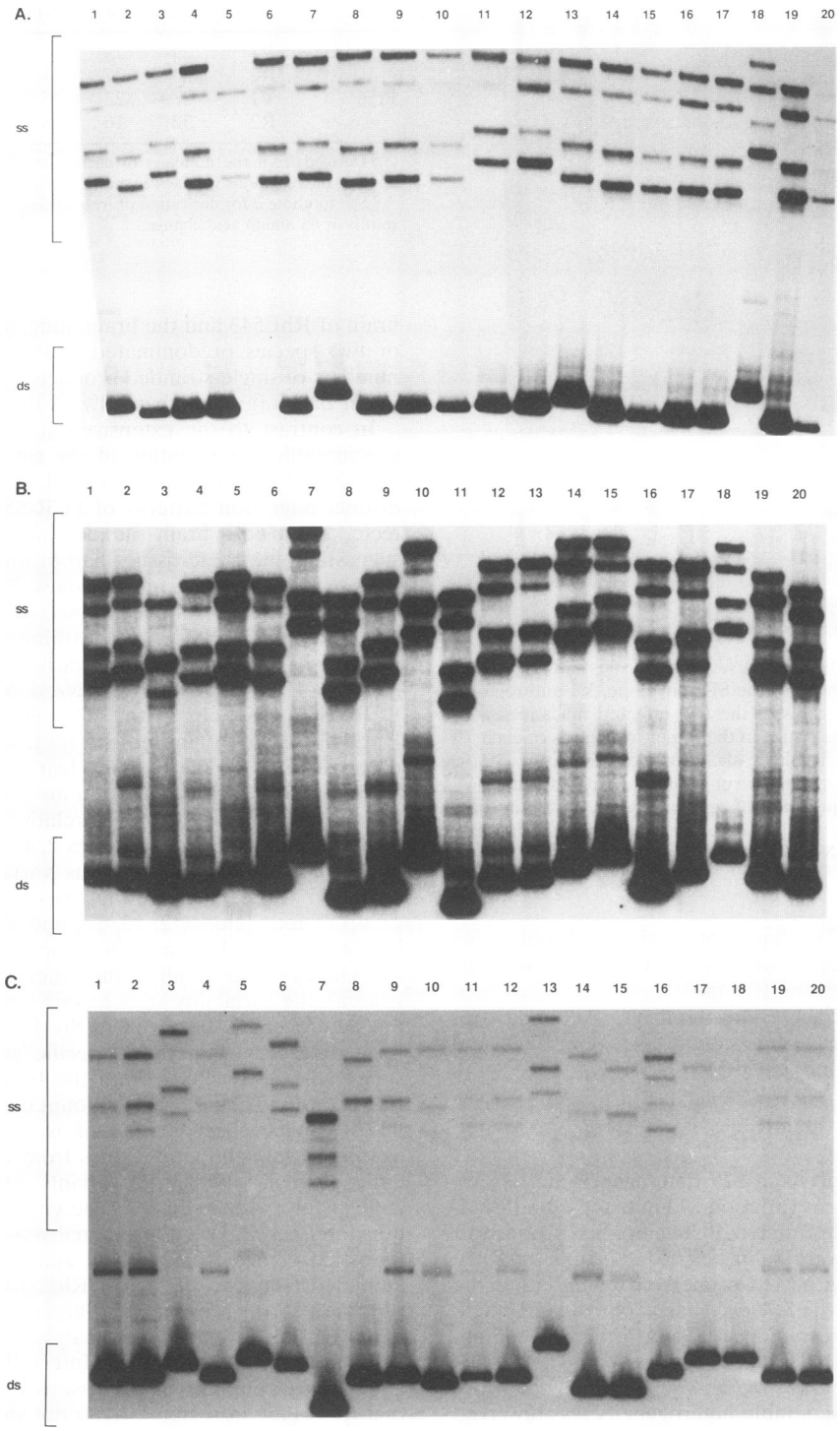


FIG. 2. PCR-SSCP analysis of clones from RhE543 tissues and PBMC. Twenty clones from the brain (A), mesenteric LN (B), and PBMC (C) were analyzed by PCR-SSCP on individual gels. Regions containing either single-stranded products (ss) or double-stranded products (ds) are indicated by the brackets on the left. There were extra single-stranded bands (other than those expected) in all V1 PCR-SSCP gels of tissue- or PBMC-derived clones. We believe that the prominent shadow bands were a second stable conformation of the DNA, which have also been noted in other PCR-SSCP reactions (19).

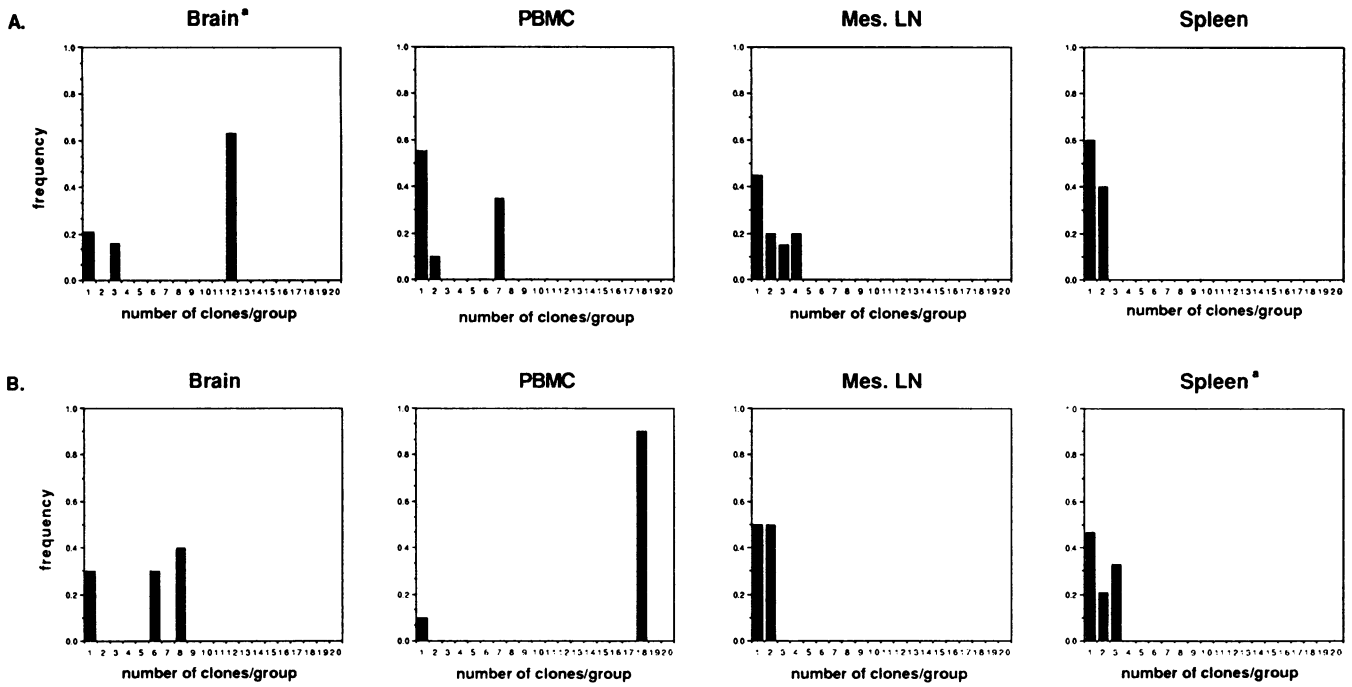


FIG. 3. Frequency distribution of groups of unique clones (columns 1) or sets of identical clones (columns 2 to 20) of the V1 region from the indicated tissue of RhE543 (A) or Pt56 (B). Frequency ($f$) = number of clones per group divided by the total number of clones analyzed. For example, one set of 12 identical clones ($f = 0.6$), one set of 3 identical clones ($f = 0.15$), and 4 unique clones ($f = 0.2$) were found by PCR-SSCP analysis of the brain of RhE543; and there were five sets of 2 identical clones ($f = 0.5$) and 10 unique clones ($f = 0.5$) found in the mesenteric (Mes) LN of Pt56. [a] Nineteen clones examined. There were significantly more groups containing two or more identical clones found in the brain of RhE543 and the PBMC of Pt56 than in the other tissues tested (chi-square analysis, $P = 0.05$).

PCR amplification generally introduces nucleotide errors into amplified products at the rate of 1 in 1,000 to 1 in 4,000 bases (13, 30). We have found in previous studies using the same reagents and conditions a PCR error rate of approximately 1 in 1,383 bases (29). Since our PCR-SSCP reactions were repeated and the same results were obtained (data not shown), variation introduced by *Taq* polymerase was less than the heterogeneity expected or observed in our amplified products.

**(i) Comparison with PCR-SSCP analysis.** Sequence analysis of the V1 region of selected SIV envelope-derived clones from RhE543 and Pt56 confirmed PCR-SSCP results. All generated sets of unique or identical clones (identified by PCR-SSCP) of the brain, mesenteric LN, and PBMC from RhE543 were grouped similarly by sequence analysis (Fig. 2, 4, and 5; some PCR-SSCP results not shown), thus demonstrating that PCR-SSCP was a reliable predictor of genetic heterogeneity in the V1 region. However, two groups of clones derived from RhE543 samples (Fig. 6A) and one group of clones from Pt56 (Fig. 6B), all of which appeared to be identical by PCR-SSCP of region 2 (data not shown), contained minor sequence variations which consisted of three or fewer nucleic acid differences. There are at least two possible related reasons for this discrepancy. First, the sensitivity of PCR-SSCP is inversely proportional to the size of the fragment analyzed by PCR-SSCP (19). Since region 2 is larger than the V1 region (284 versus 219 bp), the sensitivity of the V1 SSCP reactions may have been significantly greater than for V3. Second, there were fewer nucleotide changes in region 2 than in the V1 region. Thus, the multiple differences among V1 regions would increase the likelihood of detecting this variation.

**(ii) Types of nucleic acid and predicted amino acid changes.** Previous investigators have observed that the polymerases of SIV and HIV have a propensity for G-to-A transitions in clones derived from either PBMC (SIV-infected macaques) (9, 29, 42) or HIV-1 clones derived from cultured cells (51) or from the brain of infected individuals (34). Neither macaque in this study was infected with a molecular clone but was infected with an essentially homogeneous virus population (see Materials and Methods). Therefore, comparisons were based on nucleotide changes relative to a consensus sequence (sequences used were from Fig. 5 and 6). Unlike the results of previous investigators, nucleic acid sequences of the virus from all tissues of Pt56 contained approximately the same percentage of C-to-T transitions as G-to-A transitions (Table 1; nucleic acid data not shown).

Envelope clones were examined for potential N-linked glycosylation sites within the V1 region by comparison with a representative sequence of the virus inoculum (smH4i for RhE543 and 660 for Pt56). As shown in Fig. 4 and 5A, glycosylation patterns from the PBMC and some of the lymph node and spleen clones of RhE543 were similar to those reported previously in which N-linked glycosylation sites appeared to evolve, especially late in the disease course (42). However, the clones from the brain and some of the tissues lost potential N-linked glycosylation sites or their patterns did not change. In contrast to the results with RhE543, most clones derived from the brain, lung, mesenteric LN, and spleen of Pt56 had lost the C-terminal N-linked glycosylation site and had not gained another in the V1 region (Fig. 5B).

We also determined whether any of the changes observed in the V1 region or region 2 were tissue specific. There were no consistent deduced amino acid changes in the V1 region between different tissues from either animal. However, in

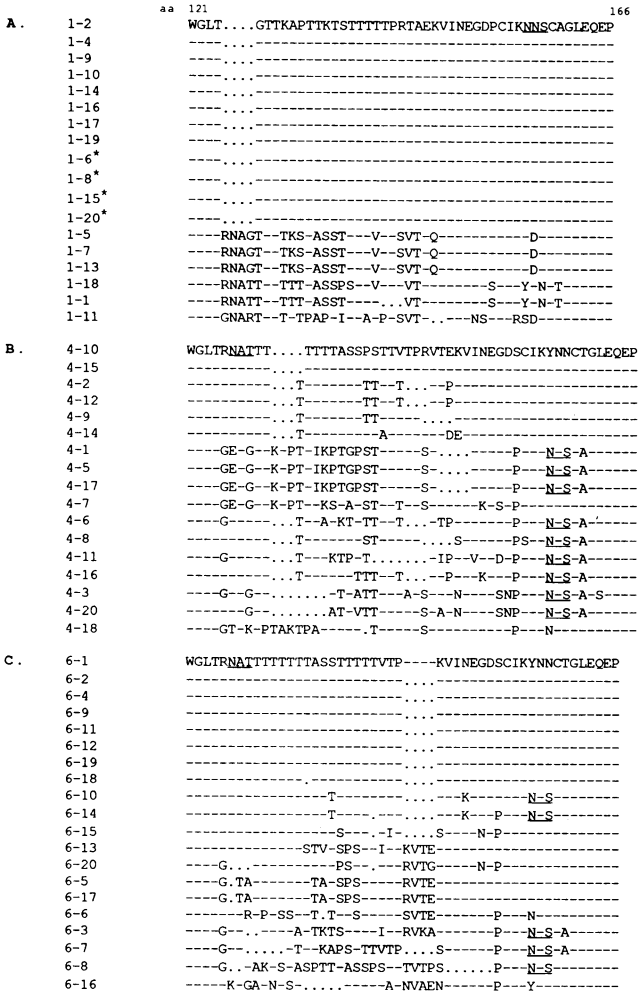

FIG. 4. Deduced amino acid heterogeneity of clones from the V1 region of RhE543 brain (A), mesenteric LN (B), and PBMC (C) envelopes. Alignments of the V1 region to the most frequent type of clone in each tissue or PBMC are shown. Amino acid residues (corresponding to psmH-4) of the beginning and end of the sequence are indicated. Potential N-linked glycosylation sites (NXS/T) are underlined. Dashes denote identity to the consensus, while dots represent gaps introduced into the sequence for optimal alignments. Clones marked with asterisks were not actually sequenced but are included on the basis of their PCR-SSCP results.

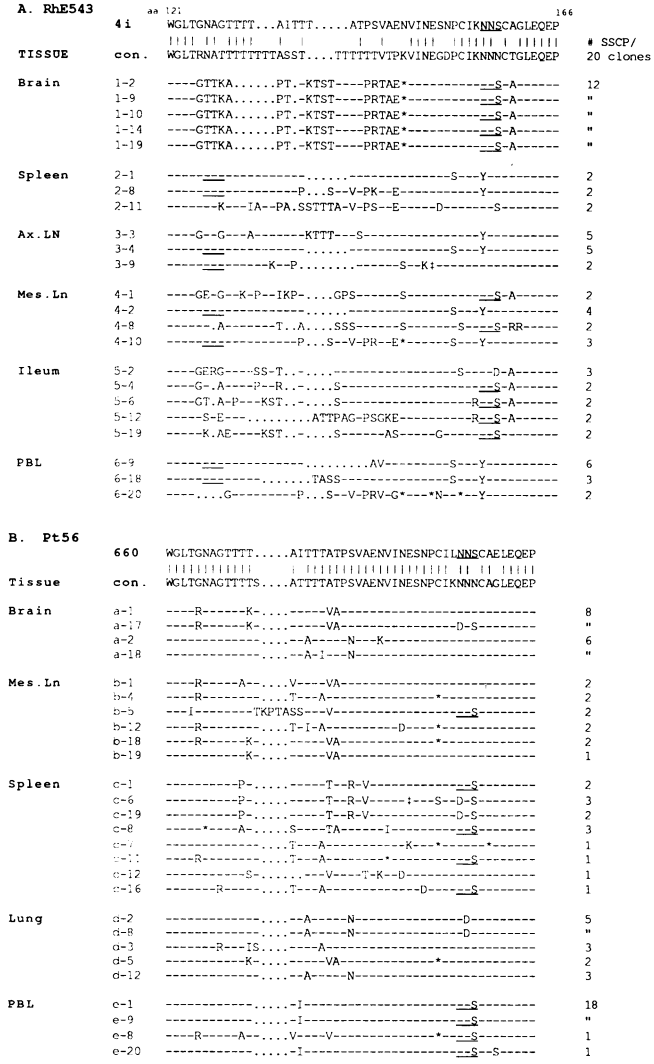

FIG. 5. Deduced amino acid heterogeneity of the V1 region of the RhE543 (A) and Pt56 (B) envelopes. A computer-assisted alignment of predicted amino acid sequences from the V1 region of selected RhE543 (or Pt56) envelope clones compared major and selected minor species (determined by PCR-SSCP) with each other and with both a consensus sequence (con.) and a representative sequence from the inoculum ($SIV_{psmH}$4i molecular clone [4i] or SIV660 clone [660]). Amino acid residues (corresponding to psmH4) of the beginning and end of the sequence are indicated. The numbers of identical clones by PCR-SSCP are listed at the right. A vertical line indicates that the clone is identical to the clone above by PCR-SSCP analysis. Potential N-linked glycosylation sites (NXS/T) are underlined. Dashes denote identity to the consensus, while dots represent gaps introduced into the sequence for optimal alignments. Amino acid changes are indicated by letters, and asterisks represent nucleic acid changes without amino acid alteration. ‡, presence of a stop codon. Ax.LN, axillary LN; Mes.LN, mesenteric LN; PBL, peripheral blood leukocytes.

agreement with PCR-SSCP analysis, two or three amino acid residues were consistently variable in region 2 of SIV clones from the brain or lung of either animal (Fig. 6; R [amino acid {aa} 375] and T [aa 383] in macrophage-rich tissues from RhE543; P [aa 335], R [aa 375], and R [aa 384] in macrophage-rich tissues from Pt56). In addition, a lysine (aa 352) residue was observed immediately downstream of the cysteine loop in the majority of the envelope clones of the virus from the spleen, axillary LN, and mesenteric LN of RhE543 but from none of the other tissues or PBMC.

## DISCUSSION

By using PCR-SSCP as a screening assay for variation within a tissue, we were able to reasonably estimate the number of envelope clones that it was necessary to sequence to accurately predict virus heterogeneity. We did not detect minor nucleotide changes when analyzing region 2, probably because of the size limitation of PCR-SSCP (19). Other alterations of the technique used here, such as restriction digestion before resolution on an acrylamide gel (19), may be necessary to further resolve single strands. Therefore, as in our previous studies using PCR-SSCP (27), this technique is useful as a

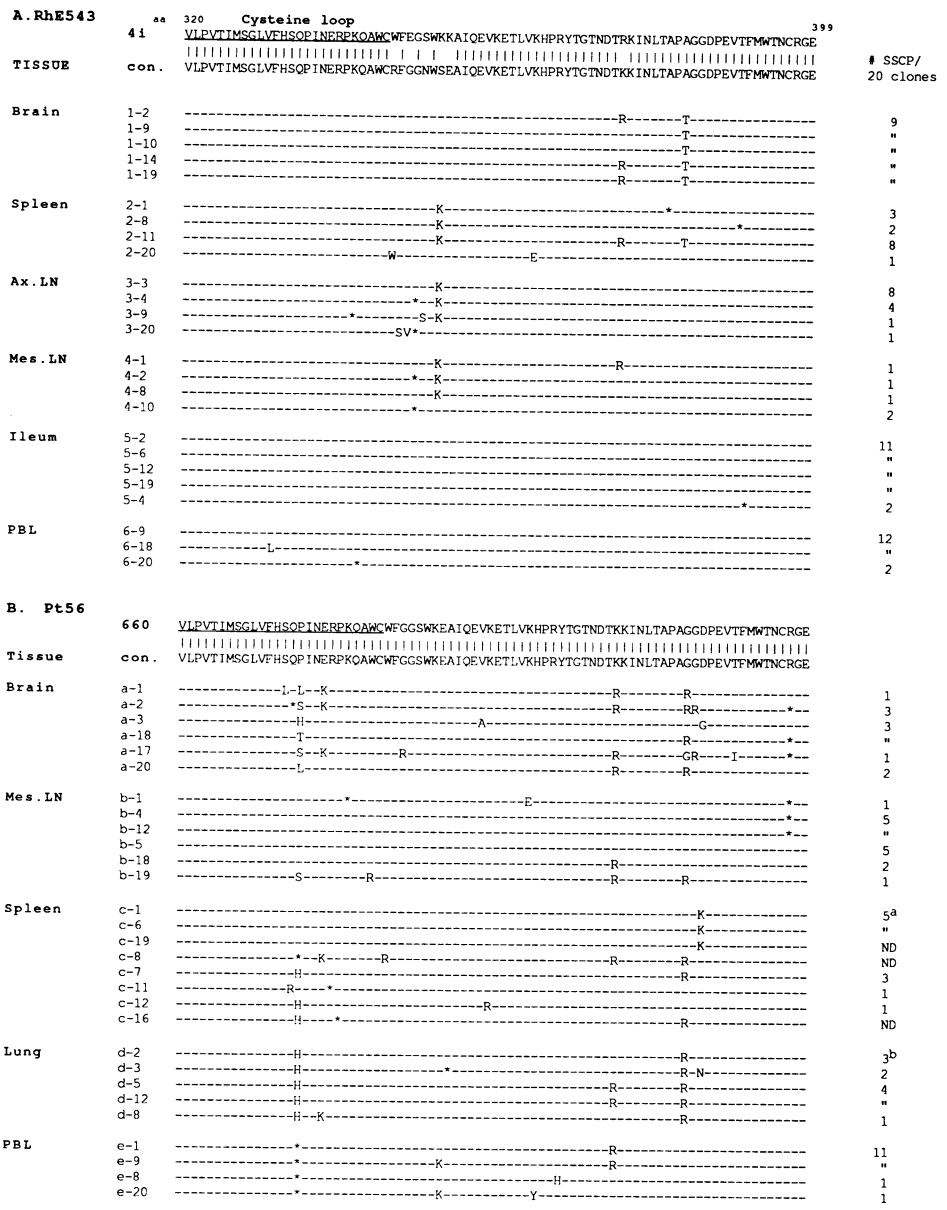


FIG. 6. Alignments of predicted amino acids in region 2 of RhE543 (A) and Pt56 (B) envelopes. Annotations are as described for Fig. 5; in addition, the SIV V3 cysteine loop (corresponding to the HIV V3 loop) is underlined. Only 17 of 20 clones from the spleen ([a]) and 18 of 20 clones from the lungs ([b]) of region 2 of the virus derived from Pt56 were examined.

rapid screening tool that, in addition to sequence analysis, provides an overall impression of genetic heterogeneity within an individual sample.

In this study, PCR-SSCP and sequence analysis were used to examine the degree of envelope heterogeneity in tissues and PBMC of $SIV_{sm}$-infected macaques. Extensive V1 region variation was observed in envelope clones from the spleens and LN of both macaques, while clones from the brain or PBMC were generally more homogeneous. Additionally, unique region 2 variants were observed in the brain and lung, tissues which would be expected to be rich in virus-infected macrophages. This region has been demonstrated in another study from this laboratory to be a critical determinant of tropism (24a). Our analysis of virus heterogeneity suggests that variation within PBMC may vastly underrepresent the spectrum of viral geneotypes within tissues of the same animal, since none of the SIV variants cloned directly from RhE543 PBMC were identical to clones from any examined tissue by either PCR-SSCP or sequence analysis. Therefore, tissues of SIV-infected macaques appear to constitute a reservoir for genetically distinct viruses with potentially different biologic and pathogenic properties. The extent of virus heterogeneity within lymphoid tissues was an unexpected finding. This contrasted with the rather limited heterogeneity observed in the brain and PBMC which has been reported for HIV-1-infected individuals (14, 43). Moreover, the type of variants also appeared to vary, depending on the tissue; the central nervous system harbored distinct virus variants. This finding is consistent with previous

studies of brain variants of both $SIV_{mac239}$ and HIV-1 (5, 14, 43). An arginine residue (aa 380) in region 2 was detected in the majority of the clones from the macrophage-containing tissues in our study. In addition, preliminary results of transfections with infectious molecular clones containing the SIV envelope from the brain of RhE543 (B-9 and B-10) indicate that this arginine residue may be required for replication in macrophages but not CEMX174 cells (10). Sequence comparisons of the entire envelope revealed that the residues which were brain specific were located throughout the envelope (unpublished results), much like the altered amino acids in macrophage-tropic $SIV_{mac239}$ (5, 37). As previously noted for HIV (40) and SIV (5, 37), this finding suggests that it is the overall envelope conformation, not specific sequence motifs, which is important in tissue or cell tropism.

The type and extent of virus variation in tissues of the two SIV-infected macaques differed in a number of respects. Analysis of variants within tissues of the strongly seropositive RhE543 demonstrated that the overall virus population had evolved considerably from the original inoculum represented by smH4. This was most evident within the first variable region of envelope, where the consensus sequence of clones from tissues differed by 39% from the inoculum. Similar evolution of region 2 was not observed. Another study of SIV-infected macaques which had strong antibody responses also indicated little heterogeneity in the V3 region of the SIV envelope (contained in region 2) (9). Amino acid substitutions, insertions, and deletions were characteristic within the V1 region of RhE543 clones. Additionally, some of the substitutions resulted in alterations in many of the potential N-linked glycosylation sites. This finding is similar to those of previous studies in SIV-infected macaques that demonstrated the acquisition of new glycosylation sites during progression to AIDS (42). In contrast to RhE543, little evolution of tissue clones of the seronegative macaque, Pt56, was observed; the consensus sequence of the V1 region differed by only 11% from the $SIV_{sm/E660}$ inoculum. In addition, the pattern of potential N-linked glycosylation sites was relatively conserved. Although the virus did not appear to have evolved, there was still considerable random variation in both the V1 region and region 2 of clones derived from tissues of this animal. This may represent the variation that would occur as a result of error-prone reverse transcription and massive uncontrolled virus replication in the absence of immune selection. These findings support previous suggestions that the V1 region is an immunoreactive epitope and possibly a portion of a neutralizing epitope (3, 42). An additional difference between the two animals was variation at the Pro-335 residue within region 2. This residue was variable only in tissues of the seronegative macaque, Pt56. Interestingly, this residue was found to be an important determinant in cellular tropism in another study in our laboratory (24a). In addition, recent evidence of extensive heterogeneity in the V3 region of SIV isolated directly from several seronegative macaque tissues also suggests that key changes that may be important in cell tropism occur in the V3 region (32). Even though the virus was $SIV_{mac239}$, one of the particular amino acid changes was also at the Pro-335 residue (32).

In conclusion, tissues of SIV-infected macaques harbor virus variants that are genetically and potentially biologically distinct from those observed in the peripheral blood. In addition, the brain of SIV-infected animals contain discrete viral sequences. These variants may serve as a continual reservoir of virus, to increase the viral burden of the host, while remaining relatively undetected by the humoral immune response. Other studies, such as a prospective analysis of the generation of tissue-specific virus variants during disease progression or an analysis of the infectivity of tissue-specific viruses, would further define the role of envelope variation within tissues in disease pathogenesis and ultimate progression to AIDS.

## ACKNOWLEDGMENTS

We thank Jennifer Martin for technical assistance and Philip Zack for pathological studies. William T. London, William Elkins, and Russell Byrum were essential in their assistance with animal studies. We also thank Robert Chanock and John Gerin for continued support and Jim Williams for helpful discussions.

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