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Complex Determinants of Macrophage Tropism in *env* of Simian Immunodeficiency Virus

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Macrophage-tropic virus variants evolved during the course of infection of individual rhesus monkeys with cloned, non-macrophagetropic simian immunodeficiency virus. Specific changes in the envelope gene (*env*) were found to be primarily responsible for the dramatic increase in the ability of the virus to replicate in macrophages. Cloned viruses differing at nine amino acid positions in *env* exhibited a more than 100-fold difference in replicative capacity for primary cultures of rhesus monkey alveolar macrophages. At least five of the nine amino acid changes contributed to macrophage tropism. These determinants were distributed across the full length of *env*, including both the gp120 and gp41 products of the *env* gene. Furthermore, the emergence of macrophagetropic variants in vivo was associated with specific pathologic manifestations in which the macrophage is the major infected cell type. Thus, major determinants of macrophage tropism reside in *env*, they can be complex in nature, and the presence of macrophage-tropic virus variants in vivo can influence the disease course and disease manifestations.

It is now well established that the human immunodeficiency virus (HIV) uses the CD4 antigen as its primary, initial receptor (18, 24, 23) and that the $CD4^+$ lymphocyte is a major target of virus replication. The decline in $CD4^+$ lymphocyte concentrations in HIV-infected people clearly contributes to the eventual development of AIDS. Less appreciated is the role of monocytes and macrophages. Numerous infected macrophages have been noted in tissues from humans that have died from HIV infection (8, 29). In fact, the tissue macrophage may be the major cell type harboring HIV in most infected individuals (10, 21, 22). Some HIV isolates in the laboratory replicate well in lymphoid and monocyte/macrophage cells, while others replicate well only in lymphoid cells (3, 11, 17). Infection of monocytes/macrophages by HIV appears to be predominantly a CD4-mediated event (2, 28). Monocyte/macrophage infection is a unifying feature of all lentiviruses, including the HIV-related lentiviruses of ungulates (visna virus, caprine arthritis-encephalitis virus, and equine infectious anemia virus) and monkeys (simian immunodeficiency virus [SIV]) (for review, see references 5, 7, and 26). However, the importance of monocytes/macrophages for the evolution of the chronic disease caused by HIV has not been defined. Is infection of monocytes/macrophages essential for persistent infection by HIV? Does infection of monocytes/macrophages influence the disease course and/or disease manifestations? What are the mechanisms that allow some isolates to enter both lymphocytes and macrophages in a CD4-dependent fashion and others to infect lymphocytes only?

SIV exhibits extensive similarity to HIV in genomic organization, gene sequences, and biological properties (5, 9). Molecularly cloned SIVmac239 causes AIDS and death in the common rhesus monkey (*Macaca mulatta*) (14). About 40% of rhesus monkeys infected with this cloned virus die with AIDS within 6 months of infection. The other 60% develop a more protracted disease course that also closely resembles AIDS in humans. Features of the AIDS-like disease include CD4 depletion, opportunistic infections, generalized lymphoid depletion, emaciation, and a unique encephalitis, all characteristic of HIV-1-induced disease in humans. The complete genetic sequence of the SIVmac239 clone has been determined (30).

In this report, we define the major genetic determinants of macrophage tropism that reside in the *env* gene. Specific amino acid changes that increase virus replication in macrophages appear to influence the particular pathologic manifestations that are observed.

MATERIALS AND METHODS

Virus. SIVmac316 was originally isolated from culture supernatants of alveolar macrophages taken by bronchoalveolar lavage from rhesus monkey 316-85 at the time of death. Virus stock was prepared following short-term propagation of virus in alveolar macrophages. SIVmac132 was isolated from the blood of rhesus monkey 132-87 near the time of death (6). Virus production in the cell-free supernatant was monitored by assay of reverse transcriptase activity (4, 25) or assay of $p27^{gag}$ antigen with a commercial antigen capture kit (Coulter Co., Hialeah, Fla.).

Cells and alveolar macrophage culture. The human T-B hybrid cell line CEMx174 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Rhesus monkey peripheral blood lymphocytes (PBL) were obtained by banding over sodium diatrizoate-Ficoll (1.077 to 1.080 g/ml at 20°C; Organon Teknika Corp., Durham, N.C.). PBL were treated with 1 μg of phytohemagglutinin per ml for 48 h, washed free of lectin, and maintained in RPMI 1640 containing 20% fetal calf serum, 10% interleukin-2 (Electro-Nucleonics, Inc., Fairfield, N.J.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Alveolar macrophages were obtained by bronchoalveolar lavage from healthy, mature rhesus macaques that were serologically negative for SIV, type D retrovirus, and simian foamy viruses. Ninety-five percent or more of the cells were

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demonstrated to be macrophages on the basis of morphologic criteria, adherence to plastic flasks, cell surface markers, and opsonizing activity, as described previously (6, 31). Macrophages were maintained in RPMI 1640 medium supplemented with 20% HIV-negative human serum (GIBCO Laboratories, Grand Island, N.Y.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin per ml at a concentration 5×10^5 cells per ml in 24- or 48-well flat-bottomed plates. The culture medium was changed twice a week.

DNA preparation and PCR amplification. Total cellular DNA was prepared from alveolar macrophages infected with SIVmac316 or SIVmac132 2 to 3 weeks after infection. Infected cells were washed with phosphate-buffered saline, lysed with NE buffer (0.15 M NaCl, 0.05 M EDTA, 0.5% sodium dodecyl sulfate, 0.2 mg of proteinase K per ml) at 50°C overnight, extracted with phenol-chloroform four times, and dialyzed against TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Four sets of oligonucleotide primers containing SIVmac239 sequences (30) (nucleotides 1→24, 5′-TG AGAATTCCTGGAAGGGATTTATTACAGTGCA-3′, and 1873←1902, 5′-GAATGTTTGTTGGGTTATACATTCTGAC AC-3′; 1819→1845, 5′-CCATACCAGTAGGCAACATTTA CAGGA-3′, and 6462←6491, 5′-GGCAATGGTAGCAACA CTTTTTACAATAGC-3′; 6437→6471, 5′-CCTCTAGAAGCA TGCTATAACACATGCTATTGTAA-3′, and 9215←9244, 5′-CTCACAAGAGAGTGAGCTCAAGCCCTTGTC-3′; 9220→ 9249, 5′-GGGCTTGAGCTCACTCTCTTGTGAGGGACA-3′, and 10261←10279, 5′-GACTCGAATTCAACTGCTAGGGA TTTTCCTGCT-3′) were used for polymerase chain reaction (PCR) amplification of SIV DNA from the total cellular DNA of the infected macrophages. From 0.1 to 1 μg of DNA was used for amplification with an automated DNA thermal cycler (Perkin Elmer Cetus, Norwalk, Conn.) for 20 to 30 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 72°C for an optimized time (1 min 5 s for 1.1-kb DNA, 2 min 30 s for 1.9-kb DNA, 3 min for 2.8-kb DNA, and 5 min for 4.6-kb DNA). An autoextension of 5 or 10 s at 72°C was added to each PCR cycle, and a 10-min final extension at 72°C was added to the last cycle. Each reaction mix contained 2 mM Mg^{2+}, 200 μM each of the four deoxynucleoside triphosphates, 0.2 μM each primer, and 2.5 U of *Taq* polymerase. Oligonucleotides used for PCR, in vitro mutagenesis, and sequencing were synthesized on a Cyclone DNA synthesizer (Biosearch, Inc.).

Molecular cloning of amplified SIV DNA and construction of chimeric provirus. Each oligonucleotide primer contained a unique restriction enzyme cleavage site. The primers corresponding to the ends of the genome contained an additional *Eco*RI site. The other primers spanned *Bam*HI (nucleotide [nt] 1849), *Sph*I (nt 6450), and *Sst*I (nt 9230) sites. Amplified SIV DNA was digested with restriction enzymes and subcloned into the pBS(−) vector (Stratagene, La Jolla, Calif.). The SIVmac239 infectious molecular clone has been subcloned as two segments, p239SpSp5′ and p239SpE3′ (15). Chimeric proviruses were constructed by recombining SIVmac316 or SIVmac132 DNA fragments into the SIVmac239 plasmids by using the unique restriction enzyme cleavage sites.

DNA transfection. Plasmids containing the 3′ and 5′ halves of the SIV genome were cleaved with *Sph*I and ligated together with T4 DNA ligase to regenerate a full genome. The ligation mixture was precipitated with ethanol and redissolved with TE. Then, 3 μg of the ligated DNA was used to transfect CEM×174 cells by the DEAE-dextran method (25). Aliquots of clarified supernatant were prepared 12 days after transfection and frozen at −70°C.

Construction of single-amino-acid substitution mutants. Site-directed mutagenesis was performed by overlap extension by PCR as described before (12, 19). The amino acid changes were introduced into the *env* of SIVmac239 as forward mutations or into the *env* of SIVmac316 as backward mutations. Specific alterations in the nucleotide sequence were introduced with a set of complementary oligonucleotides (mutagenic oligos) in which the specific change was incorporated. Mutagenic oligos for forward mutations were as follows: for Met to Ile at amino acid (aa) 165, 5′-ACAAGAGCAAATAATAAGCTGTAAA-3′ and 5′-TTT ACAGCTTAT TATTTGCTCTTGT-3′; for Lys to Glu at aa 176, 5′-CATGACAGGGTTAGAAAGAGACAAG-3′ and 5′-CTTGTCTCTTTCTAACCCTGTCATG-3′; for Asn to Asp at aa 199, 5′-GAACAAGGGAATGACACTGGTAATG-3′ and 5′-CATTACCAGTGTCATTCCCTTGTTC-3′; for Gly to Arg at aa 382, 5′-TTGACGGCTCCTAGAGGAGGAGA TC-3′ and 5′-GATCTCCTCCTCTAGGAGCCGTCAA-3′; for His to Tyr at aa 442, 5′-TCAACACTTGGTATAAAGTA GGCAA-3′ and 5′-TTGCCTACTTTATACCAAGTGTTGA-3′; for Arg to Gly at aa 751, 5′-GAAGGCAAAGAAGGAG ACGGTGGAG-3′ and 5′-CTCCACCGTCTCCTTCTTTGC CTTC-3′; for Glu to stop codon at aa 767, 5′-GGCCTTGGC AGATATAATATATTCA-3′ and 5′-TGAATATATTATAT CTGCCAAGGCC-3′. Mutagenic oligos for backward mutation were as follows: for Ile to Met at aa 165, 5′-ACAAGAG CAAATGATAAGCTGTAAA-3′ and 5′-TTTACAGCTTAT CATTTGCTCTTGT-3′; for Glu to Lys at aa 176, 5′-CATGA CAGGGTTAAAAAGAGACAAG-3′ and 5′-CTTGTCTCTT TTTAACCCTGTCATG-3′; for Asp to Asn at aa 199, 5′-GAACAAGGGAATAACACTGGTAATG-3′ and 5′-CATT ACCAGTGTTATTCCCTTGTTC-3′; for Arg to Gly at aa 382, 5′-TTGACGGCTCCTGGAGGAGGAGATC-3′ and 5′-GATCTCCTCCTCCAGGAGCCGTCAA-3′; for Tyr to His at aa 442, 5′-TCAACACTTGGCATAAAGTAGGCAA-3′ and 5′-TTGCCTACTTTATGCCAAGTGTTGA-3′; for Gly to Arg at aa 751, 5′-GGCAAAGAAAGAGACGGTGGAG AAG-3′ and 5′-CTTCTCCACCGTCTCTTTCTTTGCC-3′; for stop codon to Glu at aa 767, 5′-GGCCTTGGCAGATAG AATATATTCA-3′ and 5′-TGAATATATTCTATCTGCCA AGGCC-3′. Each mutagenic oligo pair and the outer primer pair (nt 6437→6471 and 9215←9244) were used to generate two PCR fragments having overlapping ends. A mutated *env* sequence was regenerated from the two PCR fragments by annealing at the overlapping ends where the mutation was created and subsequent PCR reaction from the outer primers (12, 19). Forward and backward mutations at aa 67 (Val/Met) and aa 573 (Thr/Lys) were made by exchanging SIVmac239 and SIVmac316-3 *env* sequences with *Sph*I (nt 6450)-*Hin*dIII (nt 6826) and *Cla*I (nt 8075)-*Nhe*I (nt 8746) fragments, respectively. Correct mutation of the viral DNA was verified by DNA sequencing (32).

RESULTS

Major determinants of macrophage tropism in *env*. SIVmac239 replicates very poorly in primary alveolar macrophage cultures derived from lung lavage samples of healthy, virus-negative rhesus monkeys (Fig. 1). Rhesus monkey 316-85 died 168 days after infection with SIVmac239, with severe weight loss, lymphoid depletion, bacteremia, giant cell pneumonia, and the characteristic granulomatous encephalitis. These latter two pathologic findings are lesions specifically associated with primary infection of tissue mac-

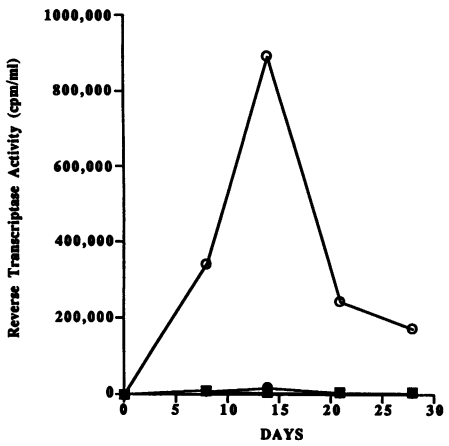

FIG. 1. Replication of SIVmac239 (●) and SIVmac316 (○) in macrophages. Virus stock containing approximately 200,000 cpm of reverse transcriptase activity was used to infect alveolar macrophages. Virus production in the cell-free supernatant was monitored by assay of reverse transcriptase activity. ■, uninfected macrophages.

rophages. SIV recovered from the lung and blood at the time of death of monkey 316-85 replicated extremely well in these primary macrophage cultures (Fig. 1) (6, 14). Thus, macrophage-tropic variants evolved from the parental SIVmac239 during the course of infection of monkey 316-85 in vivo.

PCR was used to derive DNA clones from macrophage cultures infected with this macrophagetropic derivative (SIVmac316) from the lung. Oligonucleotides spanning unique *Bam*HI (nt 1849), *Sph*I (nt 6450), and *Sst*I (nt 9230) sites were used to clone different segments of the viral genome (Fig. 2). Oligonucleotides corresponding to the ends of the genome were also used, with an additional *Eco*RI recognition tag for cloning. Recombinants were then constructed by using the infectious SIVmac239 clone and fragments derived from the SIVmac316 genome (Fig. 2).

Recombinant clones that contained the right long terminal repeat (LTR) with part of *nef* from SIVmac316 replicated well in the CEMx174 lymphoid cell line, but they did not demonstrate any significant macrophage tropism (Fig. 3A, 239/316LTR). Three independent SIVmac316 right LTR clones of slightly different sequence gave identical results (data not shown). Similarly, recombinants containing SIVmac316 sequences from nt 1 to 6450 replicated well in CEMx174 cells but they replicated poorly in the cultured macrophages (Fig. 3A, 239/316LH). In contrast, SIVmac316 sequences from nt 6450 to 9230 spanning the envelope gene imparted strong macrophage tropism (Fig. 3A, 239/316 ENV). The 239/316ENV recombinant replicated in cultured macrophages similar to virus derived entirely from cloned SIVmac316 DNA sequences (Fig. 3A). These results demonstrate that sequences within nt 6450 to 9230 are the major

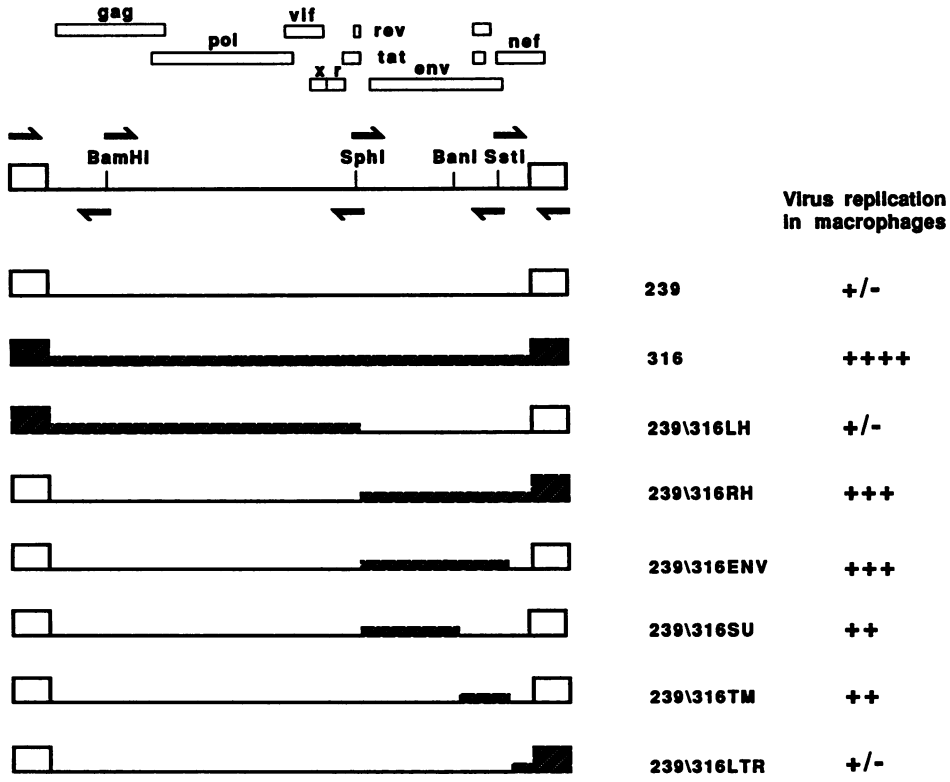

FIG. 2. Construction of recombinant viruses from SIVmac239 and amplified SIV DNA from alveolar macrophages infected with SIVmac316. PCR amplifications were performed with the primers represented by arrows. Restriction sites in SIVmac239 (*Bam*HI [nt 1849], *Sph*I [nt 6450], *Ban*I [nt 8158], and *Sst*I [nt 9230]) were used to construct the recombinant viruses. Amplified DNA was cloned by recombining fragments into the original SIVmac239 clones. The extent of virus replication in macrophages was approximated from the data shown in Fig. 3 and other experiments which are not shown.

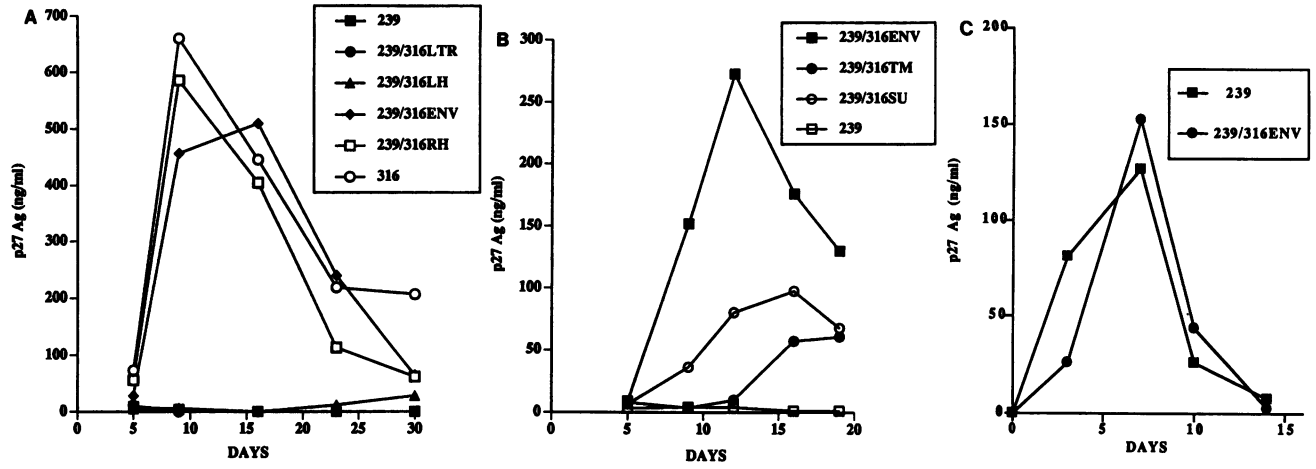

FIG. 3. Replication of recombinant viruses in macrophages (A and B) and PBL (C). The structures of the recombinant viruses are depicted in Fig. 2. (A) Virus stock containing 300 ng of p27 antigen prepared in CEM×174 cells was used to infect macrophages. Virus production in the cell-free supernatant was monitored by assay of $p27^{gag}$ antigen. (B) Virus stock containing 50 ng of p27 antigen prepared in CEM×174 cells was used to infect macrophages. (C) Virus stock containing 20 ng of p27 antigen prepared in CEM×174 cells was used to infect rhesus monkey PBL.

determinants of macrophage tropism in this system. The macrophage-tropic 239/316ENV recombinant and the parental SIVmac239 cloned virus replicated with similar kinetics and to similar extents in rhesus monkey PBL cultures (Fig. 3C). Cloned and uncloned SIVmac316 also replicated similarly to SIVmac239 in CEMx174 cells (data not shown).

A *Ban*I restriction site at nt 8158 was used to make further recombinants to gauge the relative contribution of sequences to the left and right of this site. A recombinant containing SIVmac316 sequences from nt 8158 to 9230 (239/316TM) replicated significantly better than the parental SIVmac239 in the primary macrophage cultures (Fig. 3B). This indicates that sequence changes in the TM region of the *env* gene alone can impart increased ability to replicate in macrophage cultures. A recombinant containing SIVmac316 sequences from nt 6450 to 8158 (239/316SU) replicated even better in macrophages than the 239/316TM recombinant. These results indicate that amino acids in gp120 contain major determinants of macrophage tropism. However, in repeated experiments, neither the 239/316SU nor the 239/316TM recombinant replicated as well as the full 239/316ENV recombinant (Fig. 3B). These results indicate that the determinants of macrophage tropism are complex and distributed across the full length of this region.

Amino acid changes responsible for macrophage tropism. Seven clones from the *Sph*I (nt 6450) to *Sst*I (nt 9230) sites were derived from SIVmac316-infected macrophages and sequenced (Fig. 4). Only two of these, 316-3 and 316-6, yielded infectious virus when recombined into the SIVmac239 genome. This result is consistent with the results of the DNA sequence analysis, which revealed that clones 316-1, 316-2, 316-4, 316-5, and 316-7 had obvious defects in *env* that would preclude replication competence (Fig. 4). Clones 316-3 and 316-6 had the same sequence (Fig. 4). Measurement of recombinant 239/316ENV replication, shown in Fig. 3, was done with the 316-3 clone. The DNA sequence of 316-3 revealed only 13 nt differences within nt 6450 to 9230 compared with the parental SIVmac239. Three of these changes, at nt 7890, 8268, and 8547, were silent in that they did not change the encoded amino acid in any of the known reading frames. One of the changes, at nt 9210, did not alter the encoded amino acid in the *env* reading frame (Arg), but it did change a Gly to Glu in the overlapping *nef* reading frame (Fig. 5). Mutations in *nef*, including a 182-bp deletion, were recently shown not to alter replication of the 316 clone appreciably in rhesus monkey alveolar macrophages (15), so this mutation was not considered further. The remaining nine mutations changed the encoded amino acid within *env* (Fig. 4 and 5). Of these nine changes, six were in SU (gp120) and three were in TM (gp41).

Individual site-specific mutations were then made in an attempt to define specific amino acid changes responsible for or contributing to the macrophage tropism. Nine individual forward mutants (239→316) with a single-amino-acid substitution were constructed, and nine individual backward mutants (316→239) were also made with a single-amino-acid substitution (Fig. 5). These eighteen site-specific mutants were then tested in repeated experiments for their replicative capacity in macrophages relative to that of the parental strain (Table 1 and Fig. 6). We consistently observed an influence of five of the positions on the ability to replicate in macrophages in these tests. Any single forward mutation had only a small effect by itself, consistent with multiple changes being required for the full effect. Similarly, single backward mutations did not decrease replication to anywhere near the level seen with SIVmac239. Of the five positions found to influence replication in macrophages, three are in SU (67 [Val-Met], 176 [Lys-Glu], and 382 [Gly-Arg]) and two are in TM (573 [Lys-Thr] and 767 [Glu-stop]). Changes at the other positions could also possibly contribute to macrophage tropism, but effects were not detected with the methods used.

Two of the nucleotide changes in the TM portion of the SIVmac316 *env* gene also resulted in amino acid changes in the second exon of *tat* and/or *rev* (Fig. 5). The change from A to G at nt 8854 not only changed Arg to Gly in gp41, it also changed a *rev* amino acid from Lys to Arg. The change from G to T at nt 8902, which changed a Glu to a premature stop signal in the transmembrane protein, also altered the encoded amino acid in the second exons of *tat* and *rev*. With respect to the change at nt 8854, neither the forward nor the backward mutation at this position had a detectable effect on

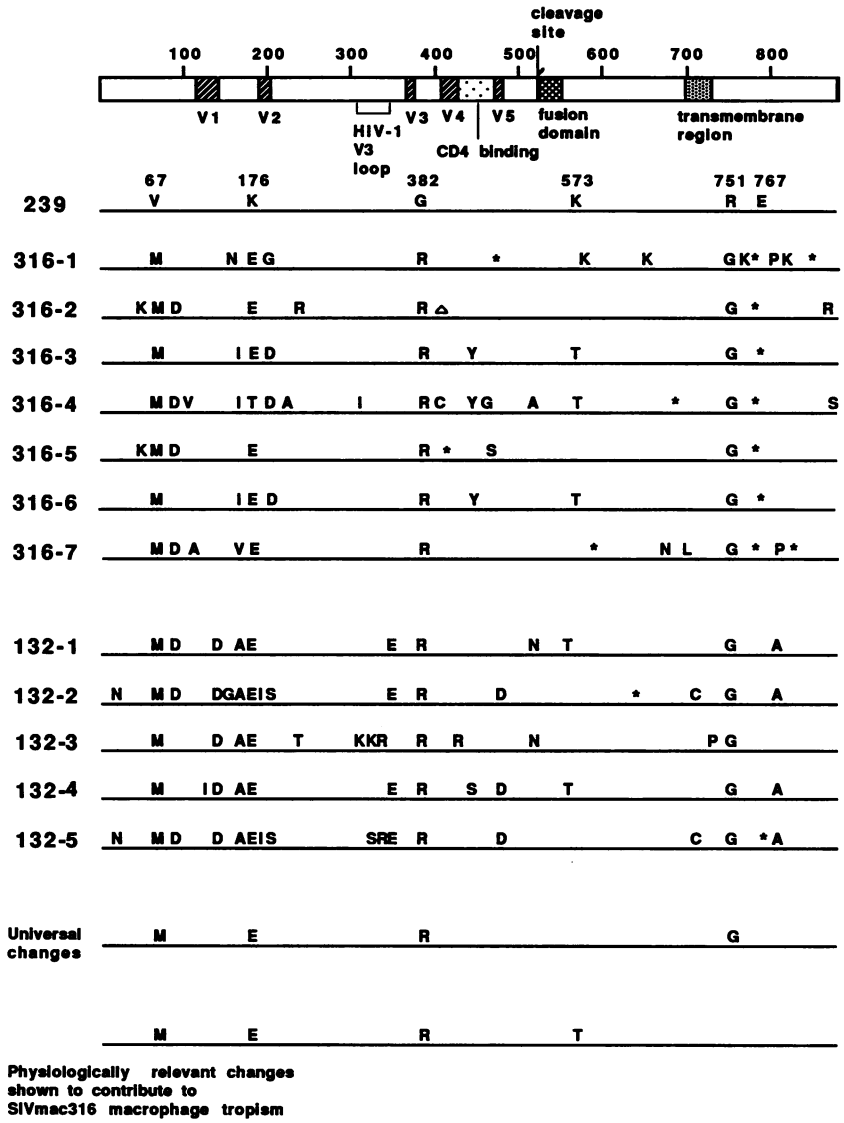

FIG. 4. Amino acid changes in *env* of macrophagetropic strains SIVmac316 and SIVmac132. SIV sequences between the *Sph*I (nt 6450) and *Sst*I (nt 9230) sites were amplified as shown in Fig. 2. The amino acid sequences were deduced from the DNA sequences. Only the amino acid changes in the *env* open reading frame are shown. Universal changes represent amino acid changes found universally in all clones of SIVmac316 or SIVmac132 in the *env* open reading frame. On the bottom line, physiologically relevant changes that contribute to SIVmac316 macrophage tropism are indicated (see text). V1, V2, V3, V4, and V5 refer to variable regions that were identified from the sequencing of SIV in persistently infected rhesus monkeys (1): V1 (nt 115 to 142), V2 (nt 188 to 201), V3 (nt 371 to 376), V4 (nt 404 to 426), and V5 (nt 473 to 478). *, stop codon. Δ, 1-bp deletion found at nt 7827 in 316-2. The locations of sequences corresponding to the HIV-1 V3 loop and the Lasky domain (CD4 binding) are also indicated.

virus replication in macrophages (Table 1). We also have evidence that the enhancing effect of the G to T change at nt 8902 (Fig. 6A, 767E→*) is due to truncation of the transmembrane protein and not to an effect on *tat* or *rev*. A similar premature truncation was created slightly upstream in SIVmac239 by changing a C to T at nt 8803. This changed a Gln at *env* amino acid 734 to a stop without affecting *tat* or *rev*. This virus replicated better than the parental SIVmac239 in macrophages and replicated very similar to the 767E→* mutant shown in Fig. 6A (data not shown). These results indicate that the enhanced replication of 767E→* in macrophages is likely due to the truncation of the transmembrane protein and not to the changes in *tat* or *rev*.

Amino acid changes in *env* of another macrophagetropic virus derived from SIVmac239. Rhesus monkey 132-87 died 180 days after SIVmac239 infection with disseminated cytomegalovirus infection, SIV-related granulomatous pneumonia, and glomerulonephropathy. SIV recovered from peripheral blood near the time of death of this animal also replicated well in primary rhesus monkey alveolar macrophage cultures, similar to SIVmac316 (6). Five clones spanning *env* were obtained from this independent macrophagetropic variant and sequenced (Fig. 4). Of the five *env* changes shown to contribute to SIVmac316 macrophage tropism, three (67V→M, 176K→E, and 382G→R) were independently observed in five of five clones from SIVmac132. Thus, three of the changes associated with and re-

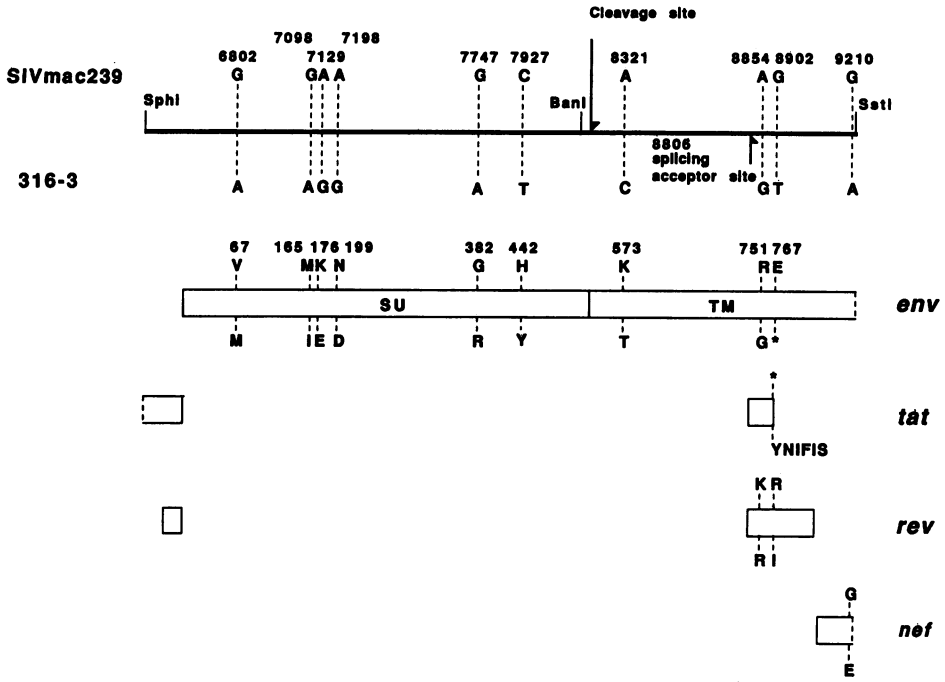

FIG. 5. Nucleotide and amino acid changes in clone 316-3. Only the nucleotide changes causing amino acid changes in *env*, *tat*, *rev*, or *nef* are shown. The numbering system is that of Regier and Desrosiers (30). SIVmac239 sequences are indicated above the horizontal lines, and SIVmac316 sequences are indicated below the horizontal lines.

sponsible for macrophage tropism emerged independently in the two animals.

DISCUSSION

Not all of the animals that die from SIVmac239 infection show the emergence of macrophagetropic variants. In fact, three of the five animals that we have studied in detail showed no evidence of macrophage-tropic variants in the peripheral blood near the time of death, showed little or no evidence of infected macrophages in tissues taken at the time of death, and showed no histologic lesions that are associated with SIV or HIV infection of macrophages (6). Granulomatous encephalitis and/or giant cell pneumonia were specifically associated with the emergence of macrophagetropic variants in rhesus monkeys 316-85 and 132-87. Thus, the appearance of macrophagetropic variants appears to influence the disease course and the specific pathologic manifestations that are observed.

We defined three amino acid changes in SU (67V→M, 176K→E, and 382G→R) and two amino acid changes in TM (573K→T and 767E→stop codon) that contribute to the macrophage tropism of SIVmac316 in vitro. Although the premature truncation of the transmembrane protein in

TABLE 1. Influence of single-amino-acid substitutions in the envelope protein on macrophage tropism[a]

aa no.	p27 antigen production							
	aa change in envelope[b]		Forward mutation[c]			Backward mutation[d]		
	SIVmac239	SIVmac316	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
67	V	M	↑	↑	↑	NT	↓	↓
165	M	I	NT	NDE	NDE	NT	NDE	NDE
176	K	E	↑	↑	↑	↓	↓	↓
199	N	D	NT	NDE	NDE	NT	NDE	NDE
382	G	R	↑	NT	↑	↓	↓	↓
442	H	Y	NT	NDE	↑	↓	↓	NDE
573	K	T	NT	↑	↑	NT	↓	↓
751	R	G	↓	NDE	NDE	NDE	NT	NDE
767	E	*	↑	↑	↑	↓	NT	↓

[a] p27 antigen production of mutant virus was compared between a forward mutant and SIVmac239 or between a backward mutant and the recombinant virus 239/316RH. Up and down arrows represent a significant increase and a 50% or greater decrease in p27 antigen production, respectively. NDE, no detectable effect. NT, not tested.

[b] Amino acid residues are shown with single letters. aa no., number of amino acids from the initiation codon. *, stop codon.

[c] Introduction of a single-amino-acid substitution into SIVmac239 *env*.

[d] Introduction of a single-amino-acid substitution into *env* of 239/316RH.

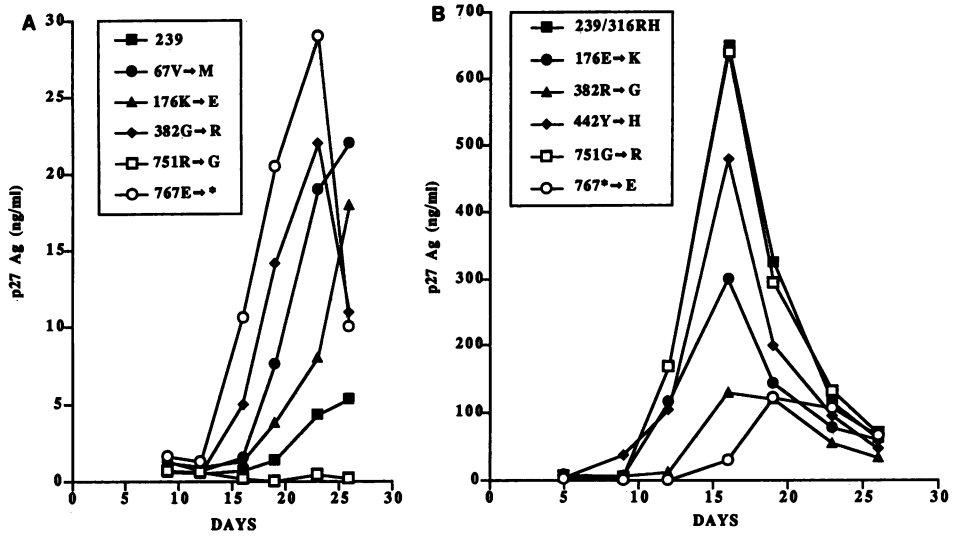

FIG. 6. Influence of single-amino-acid substitutions in *env* on macrophage tropism. $p27^{gag}$ antigen production by forward (A) and backward (B) mutant viruses in alveolar macrophages. Virus stock containing 50 ng of p27 antigen was used to infect macrophages. The numbers 67, 176, 382, 751, and 767 refer to amino acid positions in *env*. *, stop codon.

cloned 316 contributes to its ability to replicate in macrophages in cell culture, we believe that this effect is peculiar to the in vitro-cultivated virus and that it is not likely to be physiologically relevant. This belief is based on the sequence of *env* clones obtained directly from tissues that were rich in infected macrophages from monkey 316-85. Although the 67V→M, 176K→E, 382G→R, and 573K→T sequence changes that contribute to the macrophage tropism of SIVmac316 were independently observed in these tissues, premature truncation of the cytoplasmic domain of TM was not observed in these clones direct from tissues (19a). Previous studies have shown that truncation of the TM protein can enhance replication of SIVmac in some human cells and that such truncated forms can in fact be preferentially selected in these cells (20). However, the SIVmac316 strain used for these studies had never been passaged in human cells or cell lines. Thus, we have defined four amino acids in *env* (67M, 176E, 382R, and 573T) that play a major, physiologically relevant role in determining whether SIVmac316 will replicate in macrophages (Fig. 4).

Changes in addition to these four may also influence the ability to replicate in macrophages in a physiologically relevant fashion, but it may be difficult to demonstrate this unambiguously. This is true not only for other amino acid changes within *env* but also for regions outside of *env*. For example, inclusion of SIVmac316 sequences left of the *Sph*I site at nt 6450 usually resulted in slightly better replication in macrophages than when left-half sequences of SIVmac239 were used. In any event, major sequence determinants that allow vigorous SIVmac replication in macrophages and lymphocytes, as opposed to lymphocytes only, reside in *env*. Furthermore, these determinants are complex in that four or more sequence changes contribute to the maximal effect and these sequences are located in both gp120 and gp41.

Our results are similar to recent reports for HIV-1 (13, 27, 33) in that major determinants of macrophage tropism were found to map to *env*. They differ, however, in that changes in the SIVmac region corresponding to V3 of HIV-1 were not associated with macrophage tropism (Fig. 4). In one study, the V3 loop sequences of a macrophagetropic HIV-1 were able to impart maximal levels of macrophage replication to a non-macrophagetropic variant (13). Determinants of HIV-1 macrophage tropism in other studies were also located in the vicinity of V3 (27, 33). However, these results do not necessarily mean that the determinants of HIV-1 and SIVmac macrophage tropism are fundamentally different. Several studies have shown that regions in HIV-1 *env* outside of V3 can also contribute in a major way to HIV-1 macrophage tropism. For example, a 480-bp exchange spanning V3 in the reports of O'Brien et al. (27) and Shioda et al. (33) imparted only 5 to 10% of the full replicative capacity for macrophages. This suggests that the determinants of HIV-1 macrophage tropism can also be complex and that individual results will depend on the particular pair of clones being used to make the recombinants. Furthermore, it is possible that sequences in SIVmac corresponding to V3 in HIV-1 may be important for determining macrophage tropism; V3-corresponding sequences in SIVmac239 may already be nearly optimal for virus replication in macrophages. In our current study, we have precisely defined specific amino acid changes outside of V3 that contribute to the ability of the virus to replicate in macrophages.

We do not know the mechanisms by which these few defined changes in *env* can result in such a dramatic difference in ability to replicate in macrophages. Based on previous work with HIV-1 (16), virus entry is one likely possibility, but this has not yet been demonstrated for our cloned viruses. This question is particularly intriguing because infection of lymphoid cells by SIVmac239 and recombinant 239/316ENV and infection of macrophages by recombinant 239/316ENV are significantly inhibited by OKT4A monoclonal antibodies (data not shown). If the difference is indeed at the level of virus entry into macrophages, the closely matched clones described in this report will be useful in defining some of the key post-CD4-binding steps that have so far eluded investigators. The advantages of the system described in this report are the minimum number of defined sequence changes in *env* in the matched clones and the enormous difference (more than 100-fold) in ability to replicate in cultured macrophages.

The evolution of macrophagetropic variants in rhesus monkeys infected with cloned SIVmac239 was associated with the presence of specific pathologic lesions in which infected macrophages are the primary cells. These include granulomatous encephalitis and giant cell pneumonia. In this sense, the presence of macrophagetropic virus appears to influence the disease course and the specific pathologic manifestations that are observed. The importance of macrophage infection for other aspects of AIDS, including viral persistence, the chronic nature of the disease, and immune deficiency, remains to be elucidated.

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REFERENCES

1. **Burns, D. P. W., and R. C. Desrosiers.** 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. J. Virol. **65**:1843–1854.
2. **Collman, R., B. Godfrey, J. Cutilli, A. Rhodes, N. F. Hassan, R. Sweet, S. D. Douglas, H. Friedman, N. Nathanson, and F. Gonzalez-Scarano.** 1990. Macrophage-tropic strains of human immunodeficiency virus type 1 utilize the CD4 receptor. J. Virol. **64**:4468–4476.
3. **Collman, R., N. F. Hassan, R. Walker, B. Godfrey, J. Cutilli, J. C. Hastings, H. Friedman, S. D. Douglas, and N. Nathanson.** 1989. Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. J. Exp. Med. **170**:1149–1163.
4. **Daniel, M. D., Y. Li, Y. M. Naidu, P. J. Durda, D. K. Schmidt, C. D. Troup, D. P. Silva, J. J. MacKey, H. W. Kestler III, P. K. Sehgal, N. W. King, Y. Ohta, M. Hayami, and R. C. Desrosiers.** 1988. Simian immunodeficiency virus from African green monkeys. J. Virol. **62**:4123–4128.
5. **Desrosiers, R. C.** 1990. The simian immunodeficiency viruses. Annu. Rev. Immunol. **8**:557–578.
6. **Desrosiers, R. C., A. Hansen-Moosa, K. Mori, D. P. Bouvier, N. W. King, M. D. Daniel, and D. J. Ringler.** 1991. Macrophagetropic variants of SIV are associated with specific AIDS-related lesions but are not essential for the development of AIDS. Am. J. Pathol. **139**:29–35.
7. **Desrosiers, R. C., and D. J. Ringler.** 1989. The use of simian immunodeficiency viruses for AIDS research. Intervirology **30**:301–312.
8. **Eilbott, D. J., N. Peress, H. Burger, D. LaNeve, J. Orenstein, H. E. Gendelman, R. Seidman, and B. Weiser.** 1989. Human immunodeficiency virus type 1 in spinal cords of acquired immunodeficiency syndrome patients with myelopathy: expression and replication in macrophages. Proc. Natl. Acad. Sci. USA **86**:3337–3341.
9. **Gardner, M. B., and P. A. Luciw.** 1988. Simian immunodeficiency viruses and their relationship to the human immunodeficiency viruses. AIDS **2**(Suppl. 1):S3–S10.
10. **Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233**:215–219.
11. **Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, D. Skillman, and M. S. Meltzer.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J. Exp. Med. **167**:1428–1441.
12. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
13. **Hwang, S. S., T. J. Boyle, H. K. Lyerly, and B. R. Cullen.** 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science **253**:71–74.
14. **Kestler, H., T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. Daniel, N. King, and R. Desrosiers.** 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248**:1109–1112.
15. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell **65**:651–662.
16. **Kim, S., K. Ikeuchi, J. Groopman, and D. Baltimore.** 1990. Factors affecting cellular tropism of human immunodeficiency virus. J. Virol. **64**:5600–5604.
17. **Klatzmann, D., F. Barre-Sinoussi, M. T. Nugeyre, C. Dauguet, E. Vilmer, C. Griscelli, F. Brun-Vezinet, C. Rouzioux, J. C. Gluckman, J.-C. Chermann, and L. Montagnier.** 1984. Selective tropism of lymphadenopathy-associated virus (LAV) for helper-inducer T lymphocytes. Science **225**:59–63.
18. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312**:767–768.
19. **Kodama, T., D. P. W. Burns, D. P. Silva, F. Veronese, and R. C. Desrosiers.** 1991. Strain-specific neutralizing determinant in the transmembrane protein of simian immunodeficiency virus. J. Virol. **65**:2010–2018.

19a. **Kodama, T., K. Mori, D. Ringler, and R. Desrosiers.** Unpublished data.

20. **Kodama, T., D. P. Wooley, Y. M. Naidu, H. W. Kestler III, M. D. Daniel, Y. Li, and R. C. Desrosiers.** 1989. Significance of premature stop codons in *env* of simian immunodeficiency virus. J. Virol. **63**:4709–4714.
21. **Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci.** 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science **233**:1089–1093.
22. **Koyanagi, Y., S. Miles, R. T. Mitsuyasu, J. E. Merrill, H. V. Vinters, and I. S. Y. Chen.** 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science **236**:819–822.
23. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.
24. **McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson.** 1986. Binding of HTLV-III/LAV to $T4^+$ T cells by a complex of the 110K viral protein and the T4 molecule. Science **231**:382–385.
25. **Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and R. C. Desrosiers.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. J. Virol. **62**:4691–4696.
26. **Narayan, O., and J. E. Clements.** 1989. Biology and pathogenesis of lentiviruses. J. Gen. Virol. **70**:1617–1639.
27. **O'Brien, W. A., Y. Koyanagi, A. Namazie, J.-Q. Zhao, A. Diagne, K. Idler, J. A. Zack, and I. S. Y. Chen.** 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature (London) **348**:69–73.
28. **Perno, C.-F., M. W. Baseler, S. Broder, and R. Yarchoan.** 1990. Infection of monocytes by human immunodeficiency virus type 1 blocked by inhibitors of CD4-gp120 binding, even in the presence of enhancing antibodies. J. Exp. Med. **171**:1043–1056.
29. **Plata, F., B. Autran, L. P. Martins, S. Wain-Hobson, M. Raphael, C. Mayaud, M. Denis, J.-M. Guillon, and P. Debre.** 1987. AIDS virus-specific cytotoxic T lymphocytes in lung disorders. Nature (London) **328**:348–351.
30. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucle-

otide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses **6**:1221–1232.

31. **Ringler, D. J., M. S. Wyand, D. G. Walsh, J. J. MacKey, P. K. Sehgal, M. D. Daniel, R. C. Desrosiers, and N. W. King.** 1989. The productive infection of alveolar macrophages by simian immunodeficiency virus. J. Med. Primatol. **18**:217–226.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Shioda, T., J. A. Levy, and C. Cheng-Mayer.** 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) **349**:167–169.