

A Molecularly Cloned, Pathogenic, Neutralization-Resistant Simian Immunodeficiency Virus, SIVsmE543-3

VANESSA HIRSCH,^{1*} DIANE ADGER-JOHNSON,¹ BARBARA CAMPBELL,¹ SIMOY GOLDSTEIN,¹ CHARLES BROWN,¹ WILLIAM R. ELKINS,¹ AND DAVID C. MONTEFIORI²

Immunodeficiency Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Rockville, Maryland 20852,¹ and Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710²

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An infectious molecular clone of simian immunodeficiency virus SIVsm was derived from a biological isolate obtained late in disease from an immunodeficient rhesus macaque (E543) with SIV-induced encephalitis. The molecularly cloned virus, SIVsmE543-3, replicated well in macaque peripheral blood mononuclear cells and monocyte-derived macrophages and resisted neutralization by heterologous sera which broadly neutralized genetically diverse SIV variants in vitro. SIVsmE543-3 was infectious and induced AIDS when inoculated intravenously into pig-tailed macaques (*Macaca nemestrina*). Two of four infected macaques developed no measurable SIV-specific antibody and succumbed to a wasting syndrome and SIV-induced meningoencephalitis by 14 and 33 weeks postinfection. The other two macaques developed antibodies reactive in Western blot and virus neutralization assays. One macaque was sacrificed at 1 year postinoculation, and the survivor has evidence of immunodeficiency, characterized by persistently low CD4 lymphocyte subsets in the peripheral blood. Plasma samples from these latter animals neutralized SIVsmE543-3 but with much lower efficiency than neutralization of other related SIV strains, confirming the difficulty by which this molecularly cloned virus is neutralized in vitro. SIVsmE543-3 will provide a valuable reagent for studying SIV-induced encephalitis, mapping determinants of neutralization, and determining the in vivo significance of resistance to neutralization in vitro.

Human immunodeficiency virus type 1 (HIV-1) isolates vary considerably in terms of biologic properties such as cellular tropism, replicative rate, and capacity to induce cytopathic effects in tissue culture (4, 10, 15, 16, 25, 26, 29, 54, 56). Isolates from HIV-infected individuals obtained immediately after seroconversion are mostly non-syncytium inducing and macrophage tropic (56, 64), whereas later isolates are often more cytopathic and replicate preferentially in lymphocytes (4, 10, 15, 25, 29, 54). The major viral genetic determinants of macrophage versus lymphocyte tropism have been mapped to the third variable loop (V3) of HIV-1 envelope (11, 17, 29, 54, 58). The recent discovery of β -chemokine receptors as coreceptors for HIV entry may explain some of the differing tropisms of early and late HIV isolates (12, 18, 20, 21, 24). Recent studies have also demonstrated that HIV-1 strains differ in their sensitivities to neutralization in vitro (62). For example, laboratory-adapted isolates of HIV-1 that have been repeatedly passaged in T-cell lines are relatively susceptible to neutralizing antibodies and to inactivation by soluble CD4, whereas strains of HIV-1 that were isolated and propagated in human peripheral blood mononuclear cells (PBMC) are difficult to neutralize and are resistant to soluble CD4 (3, 16, 27, 47, 48). The difficulty in neutralizing these primary isolates has become an important issue for vaccine development (43), since most recombinant envelope subunit strategies used for immunization have elicited antibodies that neutralize laboratory strains but not primary isolates of HIV-1 in vitro (28, 30). The in vivo relevance of these properties is unclear since an experimental animal model for pathogenesis of HIV-1 is not available.

Simian immunodeficiency virus (SIV) infection of macaque

monkeys is a relevant model for AIDS pathogenesis studies (6, 7, 19, 33, 42, 44, 53, 57, 59, 60, 68) and for proof of concept of vaccine strategies. Although the structures of the SIV and HIV envelope proteins differ somewhat, the tropism for CD4⁺ lymphocytes and/or macrophages and the immunopathogenesis of infection of the respective hosts appear to be remarkably similar (7, 34, 42, 53, 60). As for HIV-1, determinants of SIV macrophage tropism have been localized to a region of *env* that encompasses the V3 analog (37); although the coreceptors used by SIV have not been identified, the inhibitory effects of MIP-1 α , MIP-1 β , and RANTES (CKR-5 ligands) on SIV are consistent with the use of the β -chemokine receptor family for SIV entry (14). Studies with SIVmac239 reveal that SIV tropism probably has complex determinants (1, 5, 49) since a constellation of amino acid substitutions within *env* are required to make the lymphocyte-tropic SIVmac239 replicate efficiently in macrophages in vitro (49). Although tropism for macrophages, as defined in vitro, is not required for induction of AIDS (19, 60), the ability to infect macrophages, or other nondividing cells such as Langerhans and dendritic cells, may be important for mucosal transmission (61) as well as development of SIV-induced neurologic disease (1, 49). The sensitivity of SIV challenge stocks to neutralization in vitro could also be an important aspect for modeling HIV vaccine strategies; however, many SIV challenge stocks have not been well characterized in terms of their sensitivities to neutralization. In this study, a pathogenic molecularly cloned SIV, designated SIVsmE543-3, was derived from a terminal PBMC sample (SIVsmE543 [34]) by short-term CEMx174 coculture. This report describes the molecular and biologic (in vitro and in vivo) characterization of SIVsmE543-3.

MATERIALS AND METHODS

Viruses and cells. SIVsmE543 was isolated from a terminal PBMC sample collected from rhesus macaque E543; this macaque, inoculated intravenously 3.5

* Corresponding author. Mailing address: NIAID Twinbrook II Facility, 12441 Parklawn Dr., Rockville, MD 20852. Phone: (301) 496-2976. Fax: (301) 480-2618. E-mail: vhirsch@pop.niaid.nih.gov.

years previously with uncloned SIVsmF236 (68), was euthanized due to SIV-induced neurologic disease (9, 34). Samples of lymphoid tissues and brain were snap frozen in liquid nitrogen after exsanguination and prior to perfusion with formalin. PBMC harvested at the time of necropsy were separated on lymphocyte separation medium and cryopreserved in 10% dimethyl sulfoxide and 90% fetal calf serum (FCS); thawed aliquots were used as an intravenous cell-associated virus challenge in an infectivity titration in macaques (34) and in vaccine studies (36). This cell-associated virus was highly pathogenic, with 25 to 30% of infected animals succumbing to opportunistic infections or wasting and encephalitis by 6 months after inoculation (34, 36). Virus was isolated by short-term cocultivation (14 days) of PBMC collected at the time of necropsy with CEMx174 cells (SIVsm/E543), and genomic DNA was isolated from cultures expressing supernatant reverse transcriptase (RT) activity (9).

Molecular cloning. A bacteriophage lambda library was constructed from genomic DNA extracted from the short-term E543 PBMC-CEMx174 coculture described above. Sucrose gradient size-selected (9- to 20-kb) *Hind*III-digested genomic DNA was ligated into *Hind*III-digested lambda DASH II (Stratagene, La Jolla, Calif.). Approximately 1 million recombinant plaques were screened by hybridization with a SIVsmH4 *gag* probe; four full-length clones were identified and plaque purified. Lambda DNA from each of these clones was transfected by the DEAE-dextran-mediated method into CEMx174 cells, and the culture supernatants were monitored for RT activity. Based on transfection results, one clone was infectious and was designated SIVsm/E543-3. The entire genome of this clone was subcloned into the *Sph*I and *Hind*III sites of the plasmid vector pGEM7Zf (Promega, Madison, Wis.). To facilitate sequence analysis, the 5' and 3' portions of the provirus were subcloned into pGEM-7Zf (+), using the lambda cloning site *Hind*III and an internal *Csp*45I restriction site. The complete genome was sequenced in both strands, using the dideoxy-chain termination method with T7 DNA polymerase (United States Biochemical Corp., Cleveland, Ohio). Sequences were analyzed by using the PCGene programs, Intelligenetics (Mountain View, Calif.) programs (GENALIGN and SEQ), and CLUSTAL V.

PCR amplification and cloning. Total cellular DNA (100 ng) extracted from the brain of rhesus macaque E543 and a terminal PBMC sample were used in nested PCRs to amplify the entire envelope gene (9), using Perkin-Elmer Cetus reagents and thermocycler. Primers used to amplify the *env* gene were as follows, with underlined nucleotides indicating restriction enzyme sites introduced to facilitate cloning, italicized nucleotides indicating random sequence, and numbers in parentheses indicating the position of the last nucleotide of the primer within the genome: EnvR1F, 5'TGGGATGTCTTGGGAATCAGCTGCTTA (6588); EnvR1R, 5'CTTTCTTGGCTGAATTTGTGCTTCTTC (8596); EnvR2F, 5'TACTTCGAAGGATCCACCATGGGATGTCTTGGGAATCAG; and EnvR2R, 5'GTTGCATGCGGATCCCTACAAGAGAGTGAGCTCAAG.

A fraction of the products was analyzed on a 0.9% agarose gel, and the remainder was digested with *Csp*45I and *Sph*I and cloned into the plasmid pGEM-7Zf (+) vector (Promega). Four entire envelope clones derived from each source (B-1, B-9, B-10, and B-19 from the brain and P-2, P-20, P-21, and P-4 from PBMC) were sequenced by using dideoxy-chain termination with T7 polymerase (United States Biochemical).

Infectivity studies using cell culture and macaques. Cell-free virus stocks were aliquoted from CEMx174 cells transfected with the SIVsmE543-3 molecular clone and used to assess in vitro infectivity in macaque PBMC and macaque monocyte-derived macrophages (MDM) from a simian retrovirus type D (SRV)-, simian T-lymphotropic virus type 1 (STLV-1)-, and foamy virus-free pig-tailed macaque. PBMC (5×10^6) were pelleted after culture for 4 days in RPMI 1640 containing 10% FCS, 1% interleukin-2, and 5 μ g of phytohemagglutinin, resuspended in 1 ml of cell-free virus, and incubated at 37°C for 1 h with intermittent mixing. PBMC were subsequently washed three times with Hanks balanced salt solution (HBSS) and resuspended in RPMI 1640 as above but lacking phytohemagglutinin. Culture supernatants were periodically monitored for RT activity over a 4-week period, harvested at peak activity, filtered through a 0.45- μ m-pore-size filter, and cryopreserved in liquid nitrogen in 1-ml aliquots. MDM cultures were derived by plating approximately 5×10^6 PBMC into a 48-well tissue culture plate (31) in 0.5 ml of RPMI 1640 containing 10% FCS and 10% normal macaque, heat-inactivated serum. MDM were allowed to adhere for 5 days, then washed gently with HBSS to remove nonadherent lymphocytes, and infected 24 h later, after an additional wash with HBSS. Infection was performed by incubation of 0.5 ml of cell-free virus stock on MDM for 1 to 2 h followed by extensive washing to remove residual virus. Culture supernatants were subsequently monitored for RT activity for approximately 3 weeks.

To assess the in vivo infectivity and pathogenicity of the SIVsmE543 clone, four juvenile, wild-caught, 4- to 5-kg pig-tailed macaques (*Macaca nemestrina*) were inoculated intravenously. The cell-free virus stock used was derived by transfection of CEMx174 cells and subsequent infection of PBMC from an SIV-, foamy virus-, SRV-, and STLV-1-free pigtailed macaque. Two additional macaques (PT581 and PT587) were mock infected and subjected to parallel sample collection for the first 3 months after inoculation as a control for lymphocyte subset changes. These animals remained clinically healthy and subsequently were used in other SIV infection studies. Animals were subsequently monitored by virus isolation from PBMC (5×10^6) by cocultivation with CEMx174 cells at 1, 2, 4, 8, 16, 24, and 33 weeks and every 2 months subsequently, virus isolation on disrupted lymph nodes (5×10^6 cells) and in situ hybridization (ISH) of lymph node biopsies collected at 2, 4, and 16 weeks postchallenge, antigen capture

TABLE 1. Comparison of predicted amino acid sequences of SIVsmE543 proteins and other SIVsm and SIVmac proteins

SIVsmE543-3 protein	% Similarity with protein from:				
	SIVsmH4	SIVsmmPBj	SIVmac239	SIVmne	SIVstm
Gag	98.6	94.0	88.9	91.9	89.9
Pol	99.0	97.4	92.1	91.9	91.2
Vif	96.7	91.1	82.2	83.2	68.3
Vpx	93.7	89.3	91.9	92.9	88.4
Vpr	97.0	93.1	85.1	89.1	84.1
Tat	94.6	86.2	64.1	64.9	67.9
Rev	91.0	85.1	67.3	68.9	70.5
Env	92.6	87.3	81.8	82.3	81.9
Nef	94.8	79.9	73.8	74.5	76.1

detection of viral antigen in plasma (Coulter Corp., Hialeah, Fla.), lymphocyte subset analysis (CD4, CD8, CD2, and CD20) by fluorescence-activated cell sorting on whole heparinized blood samples (33), complete blood counts, Western blot detection of SIV-specific antibody, and neutralization assay detection of SIV-specific antibody. Western blots were performed by using as the antigen SIVsmH4 virus produced by transfection of CEMx174 cells, pelleted through 10% sucrose with ultracentrifugation. Animals were also monitored clinically for weight gain, appetite, and overall clinical condition. Animals were euthanized and subjected to a complete necropsy if they developed diarrhea unresponsive to specific and supportive therapy or lost more than 10% of their body weight.

Construction of envelope chimeras. Chimeric clones of the SIVsmE543-3 genome were constructed by substitution of a 2.6-kb *Bsm*I-to-*Sac*I fragment digested from the envelope clones (B-9 and B-10) generated by PCR amplification from total genomic DNA of the brain of E543. These two chimeras (SIVsmE543-3/B-10 and -B-9) were confirmed by limited sequence analysis of the envelope and transfected into CEMx174 cells to assess infectivity. One chimera, SIVsmE543-3/B-10, appeared to be biologically active, and all further assessments were performed with cell-free supernatants collected from this transfection. The relative infectivities of the clones for CEMx174 cells, macaque PBMC, and macaque macrophages were assessed by using techniques described above for infectivity studies with cell culture and macaques. Relative virus expression was assessed by quantitation of RT activity with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Neutralization assay. SIV neutralization was measured as described previously (44) except that CEMx174 cells were used in place of MT-2 cells as targets for infection. Briefly, cell-free virus (50 μ l containing 0.5 to 1 ng of p27) was added to multiple dilutions of test plasmas in 100 μ l of growth medium in triplicate wells of 96-well microdilution plates and incubated at 37°C for 30 min before addition of CEMx174 cells (10^5 cells in 100 μ l per well). Cell densities were reduced, and medium was replaced after 3 days of incubation. Incubation was continued until virus-induced syncytium formation and cell killing were observed microscopically in wells incubated in the absence of test plasma. Neutralization was measured by staining viable cells with Finter's neutral red in poly-L-lysine-coated plates as described previously (44). Neutral red uptake by CEMx174 cells is linear from 3.1×10^4 to 5×10^5 viable cells/well, corresponding to A_{540} values of 0.25 to 1.6. Percent protection was calculated by the difference in absorption between test wells (cell plus plasma plus virus) and virus control wells (cells plus virus), divided by the difference in absorption between cell control wells (cells) and virus control wells. Neutralization titers are given as the reciprocal of the plasma dilution at which 50% of cells remained viable after extensive virus-induced cell killing had occurred in the absence of antibody.

Virus stocks for neutralization assays were produced in H9 cells (SIVsmB670, SIVmac251, SIVsmH4, and SIVsmE543-3) or CEMx174 cells (SIVsmE543-3). Virus-containing culture supernatants from acutely infected cells were prepared by filtration through 0.45- μ m-pore-size filters and stored in aliquots at -70°C until use. An additional stock of SIVsmE543-3 used for neutralization assays included the rhesus PBMC-grown infection stock described above. Virus concentrations were determined by SIV p27⁹⁸ antigen capture as described by the supplier (Coulter Immunobiology, Hialeah, Fla.).

ISH. ISH was performed essentially as described previously for SIVagm (38) with digoxigenin-labeled antisense RNA probes generated by SP6 or T7 polymerase transcription from 1- to 2-kb subclones spanning the entire SIVmac239 genome (a gift of S. Gartner and P. Racz) on formalin-fixed, paraffin-embedded tissues. To confirm the specificity of the signal, digoxigenin-labeled sense RNA probes were used on selected positive tissues; no specific hybridization was observed with the sense probe preparation. Briefly, tissues were hybridized with 1.75 ng of riboprobe per ml at 52°C overnight, washed sequentially in 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-50% formamide solution and 2 \times SSC, and treated for 30 min in an RNase solution (RNase T₁ and RNase A in 2 \times SSC) at 37°C. The slides were blocked with buffer containing 2% horse serum, 150 mM NaCl, and 100 mM Tris (pH 7.4) for 1 h. Following blocking, the slides were incubated for 1 h with sheep anti-digoxigenin-alkaline phosphatase

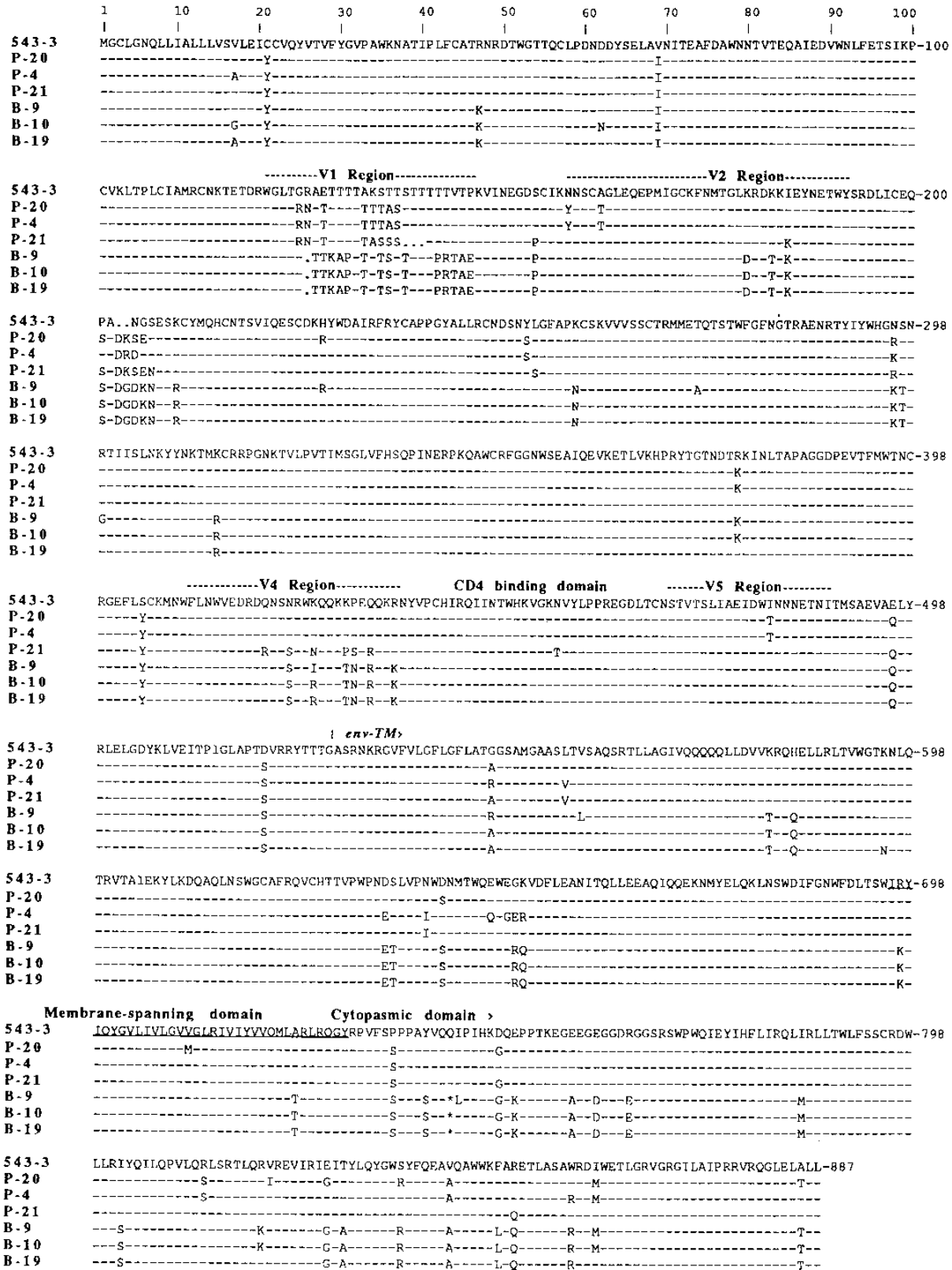


FIG. 1. Amino acid alignment of envelope genes of the full-length infectious clone SIVsmE543-3 and six envelope subclones, three derived from the brain (B-9, B-10, and B-19) and three cloned directly without coculture from PBMC (P-4, P-20, and P-21). The predicted amino acid sequence of the envelope of SIVsmE543-3 clone is shown at the top in single-letter code, with dashes below indicating identical amino acids in aligned sequences, amino acid substitutions indicated, gaps introduced to maximize alignment shown by dots, and an asterisk to indicate a premature stop codon. The various variable and functional regions of the envelope gene (V1, V3, V4, V5, CD4 binding domain, and cytoplasmic domain) are indicated.

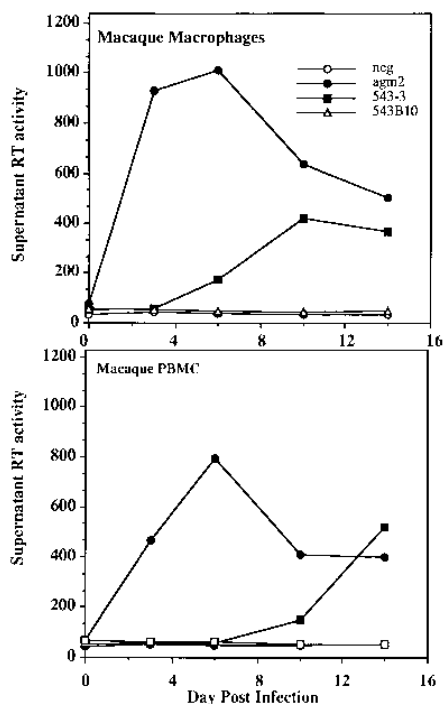


FIG. 2. Sequential supernatant RT activity following infection of macaque PBMC and MDM by SIVsmE543mc (543-3) and the 543 brain envelope chimera (543B10). SIVagm9063 was used as a positive control since it efficiently infects both primary cell types. PBMC and macrophages were infected with equivalent amounts of virus as described in Materials and Methods; cell-free supernatants were collected sequentially and assayed for RT activity, which was quantitated with a PhosphorImager (Molecular Dynamics).

conjugate at a 1:500 dilution, and incubated overnight with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP; Vector Laboratories, Burlingame, Calif.) substrate at room temperature overnight. Slides were counterstained with nuclear fast red, dehydrated, and coverslipped. Tissues examined by ISH were axillary, inguinal, bronchial, and mesenteric lymph nodes, spleen, thymus, stomach, ileum, ileocecal junction, colon, brain (two sections), thoracic and lumbar spinal cord, lung, kidney, and liver.

Nucleotide sequence accession number. The entire SIVsmE543-3 sequence has been deposited in GenBank under accession number U72748.

RESULTS

Sequence comparison to other SIV molecular clones. The genomic organization of the SIVsmE543 molecular clone was essentially identical to that observed previously for other molecular clones of SIVmac and SIVsm (31, 35). The virus encoded *gag*, *pol*, and *env* as well as *vif*, *vpx*, *vpr*, *tat*, *rev*, and *nef*. Similarities of various predicted proteins to those of other molecular clones in this subgroup are shown in Table 1. As expected, the 543 clone was most highly related to SIVsmH4 (31); this latter clone was derived from SIVsmF236-infected H9 cells that were the source of the SIV stock used to infect rhesus macaque E543 (34). The most divergent genes in the comparison of SIVsmH4 and SIVsmE543-3 were the envelope and *rev* genes, with 92 and 91% identity in amino acid sequence, respectively. The next most closely related SIV was the acutely lethal SIVsmmPBj molecular clone. In contrast to SIVmac molecular clones, the E543 clone encoded full-length *env* and *nef* genes.

Envelope clones from brain and PBMC. As observed previously in SIV (1, 9) and HIV (23, 41, 52) infections, distinct populations of virus were found in the brain of E543 (9). The virus genotypes cloned from the brain differed from the 543-3 molecular clone and other independent clones derived from uncultured PBMC at scattered residues throughout the envelope.

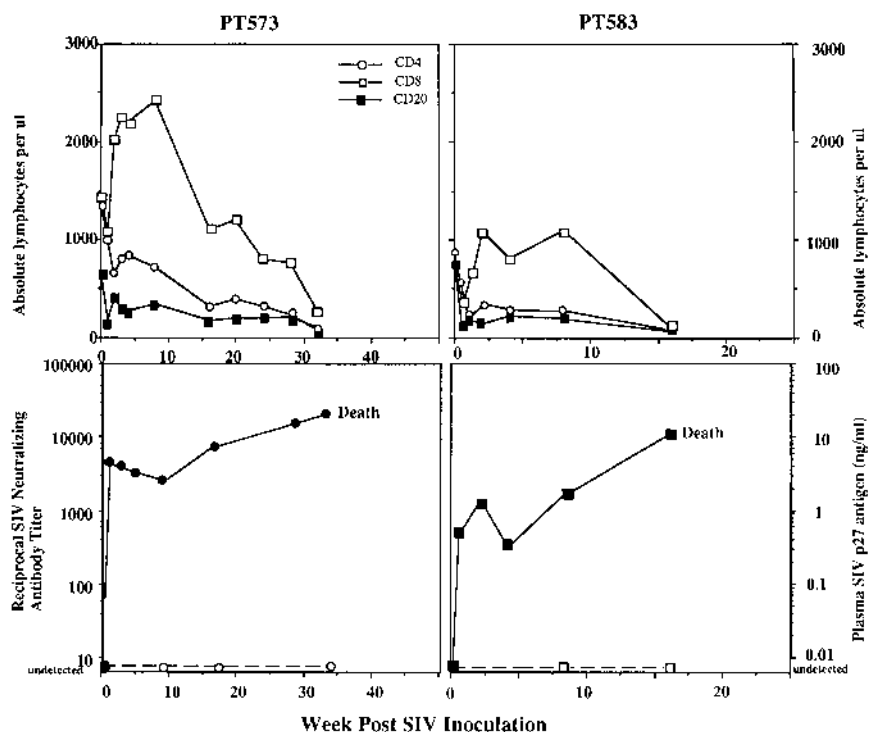


FIG. 3. Sequential alterations in lymphocyte subsets in the peripheral blood (CD4, CD8, and CD20) observed in two macaques (PT573 and PT583) that rapidly progressed to AIDS following inoculation with SIVsmE543-3 virus (top graphs). Concurrent sequential plasma p27 antigen levels (filled symbols) and SIVsmE543-3 neutralizing antibody titers (open symbols) are depicted in a double-Y format on the bottom graphs.

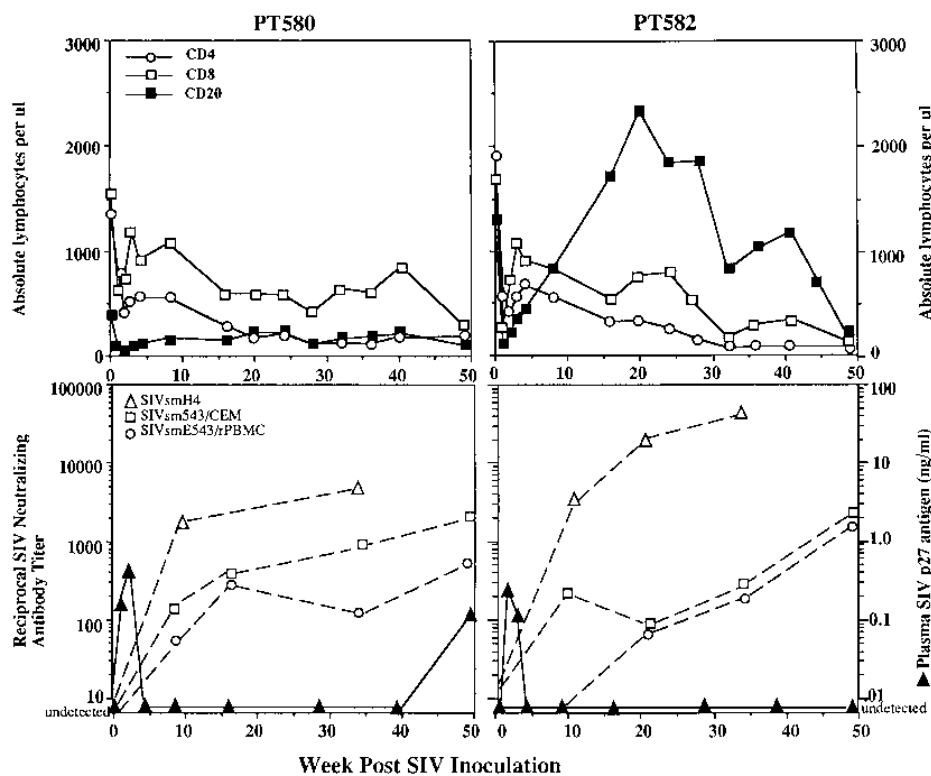


FIG. 4. Sequential alterations in lymphocyte subsets in the peripheral blood (CD4, CD8, and CD20) observed in PT580 and PT582, two macaques that developed SIV-specific antibody and progressed more slowly to AIDS, are shown on the top graphs. Concurrent sequential plasma p27 antigen levels and neutralizing antibody titers are shown in the bottom graphs, depicted on a double-Y graph format with a logarithmic scale with the minimum level of sensitivity of detection indicated. Filled symbols indicate plasma antigen which was detectable in these animals during primary viremia and again just prior to death in PT580 plasma samples. The open symbols indicate neutralizing antibody titers for SIVsmE543-3 grown in CEMx174 cells (open squares), SIVsmE543-3 grown in rhesus macaque PBMC (rPBMC; open circles), and SIVsmH4 (open triangles). In both animals, the virus strain used for inoculation (SIVsmE543) was considerably more resistant to neutralization; thus higher titers were achieved to a related, neutralization-sensitive SIV strain (SIVsmH4).

lope gene, consistent with the differences observed between SIVmac239 and macrophage-tropic variants arising in vivo (1, 49). A concentration of substitutions in the V1 region was also observed, as noted previously in studies of SIV in vivo evolution (1, 9, 37, 49, 51). As shown in Fig. 1, clones derived from the brain of this macaque encoded a prematurely truncated envelope gene due to an in-frame stop codon in the position observed in SIV after passage in human cell lines (32, 40). A clustering of five amino acid substitutions unique to brain-derived clones were observed in the portion of *env* open reading frame overlapping *rev* and *tat* (Fig. 1), resulting in eight and two amino acid substitutions, respectively, in *Tat* and *Rev*. Since this portion of the *env*-transmembrane region (TM) would not be expressed due to premature termination, such variation within the second coding exon of *tat* and *rev* could be of biological significance. Sequence analysis of additional independent *env* clones (9) from the brain revealed a predominance of envelopes with premature truncation (i.e., in 14 of 20 clones). In contrast, none of 20 clones derived from PBMC of this animal contained in-frame stop codons in the TM portion of *env*. The length of time since inoculation (over 3 years), the lack of envelope truncations in two representative clones from the inoculating virus (27), and the overall divergence (8%) of brain-derived *env* sequences from those in the blood are consistent with de novo generation and selection of these truncated variants. The biological significance of these brain variants was evaluated by constructing chimeras of SIVsmE543-3 in which the majority of *env* (from *BsmI* near the signal peptide

to *SstI* in the end of the cytoplasmic domain) was exchanged with two representative brain clones, B-9 and B-10 (Fig. 1). These chimeras were isogenic in other respects except for previously mentioned substitutions in the second exon of *Rev* and *Tat* and seven substitutions in the amino-terminal 68 amino acids of *Nef*.

Biological activity and tropism of SIVsmE543-3. Transfection of CEMx174 cells with either SIVsmE543-3 or SIVsmE543-3/B-10 (a chimera expressing the predominant SIV envelope in brain) by DEAE-dextran methods resulted in productive infection with supernatant RT activity and characteristic syncytium formation (data not shown). The highly related SIVsmE543-3/B-9 clone was inactive in this assay and was not evaluated further. The tropism of parental SIVsmE543-3 and the brain *env* chimera was investigated by cell-free infection of macaque PBMC or macaque MDM. As shown in Fig. 2, the E543-3 cloned virus replicated in both macaque PBMC and MDM. The positive control virus used in this assay, SIVagm9063-2 (38), replicated consistently to higher levels and with more rapid kinetics. The chimeric clone expressing the predominant envelope gene in the brain did not replicate appreciably in primary macaque cells. Previous studies of SIV clones with similar TM truncations following adaptation to human cell lines have also demonstrated preferential replication in human cell lines, such as H9, rather than macaque PBMC. Therefore, although this finding was not unexpected, it does not explain presence of this variant in the brain of E543. Potentially other genes (such as *vpx*, *vpr*, and *nef*) or long

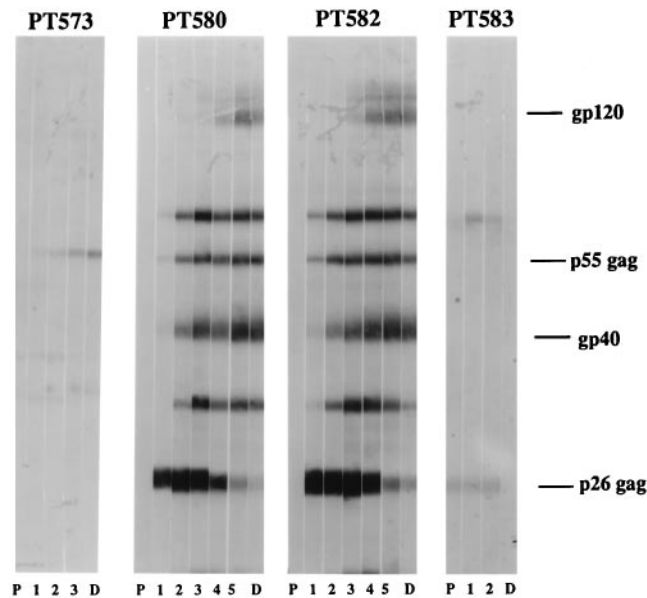


FIG. 5. Western blot reactivity of sequential plasma samples from SIVsmE543-3-infected macaques. Each strip represents a different plasma sample taken preinoculation (lane P), 4 weeks (lane 1), 8 weeks (lane 2), 16 weeks (lane 3), 32 weeks (lane 4), 52 weeks (lane 5), and death (lane D). Samples from two animals (PT573 and PT583) contained no detectable SIV-specific antibodies. In contrast, SIV-specific antibody is detectable in 8-week samples from both PT580 and PT582, with increasing reactivity to envelope proteins throughout the subsequent disease course but a decline in reactivity of Gag-specific antibody toward death.

terminal repeat variants in virus within the brain are required to act in concert with the envelope. Also, since only PBMC and MDM were evaluated, this variant could have preferential tropism for other brain-derived cell types, such as endothelial cells or glial cells, which were not evaluated in this study.

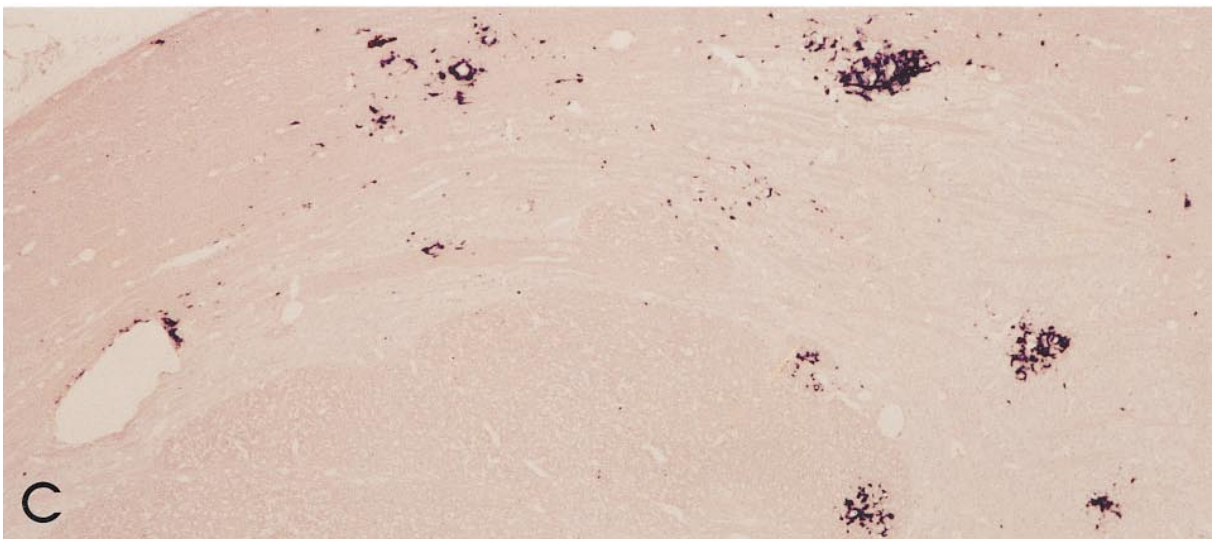
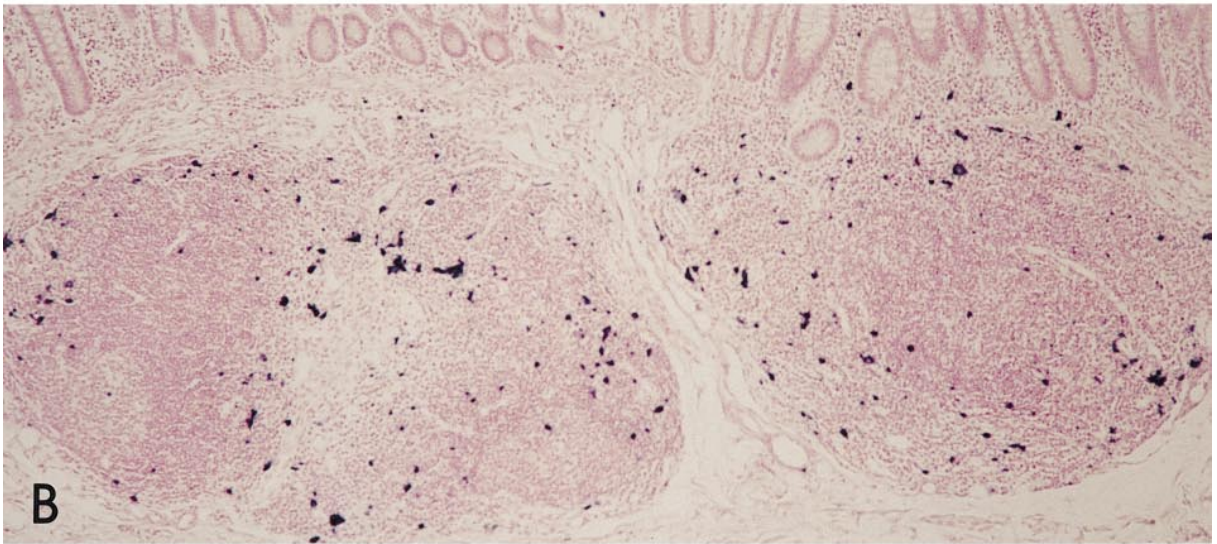
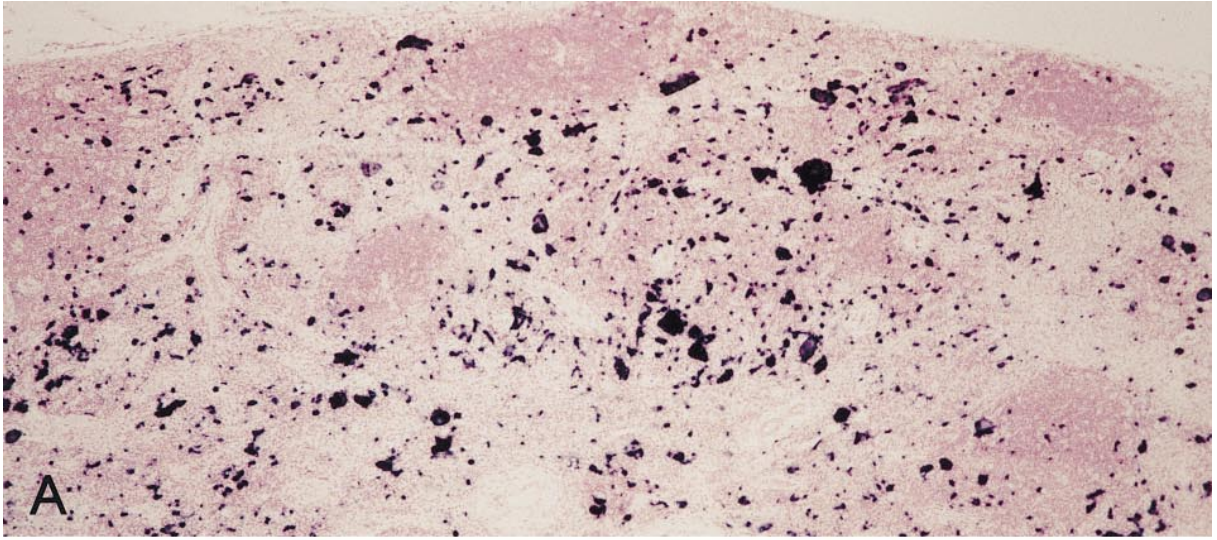
Molecularly cloned SIVsmE543 is infectious and pathogenic in macaques. Four pig-tailed macaques (*M. nemestrina*) were inoculated intravenously with approximately 1,000 50% tissue culture infective doses of cell-free SIVsmE543-3 generated by infection of PBMC from a specific-pathogen (SRV, simian foamy virus, and STLV-1)-free pig-tailed macaque. All four inoculated macaques became persistently infected, as evidenced by consistent virus rescue from sequential PBMC samples (at 1, 2, 4, and 8 weeks and every 8 weeks thereafter) and lymph node biopsies obtained at 2, 4, and 16 weeks (data not shown). Each animal exhibited an early (2-week) peak of plasma viral p27 antigen, which persisted and increased in two of the animals (PT573 and PT583 [Fig. 3]) but resolved in two other animals (PT580 and PT582 [Fig. 4]) by 4 weeks postinoculation. These latter two animals developed SIV-binding antibodies by 2 weeks, as evidenced by SIV Western blotting (Fig. 5) and HIV-2 whole-virus enzyme-linked immunosorbent assay, achieving peak titers of 1:12,800 and 1:25,600 by 8 weeks (data not shown). In contrast, the animals with persistent antigenemia had no detectable SIV-specific antibody as measured by Western blot or neutralization assay. However, one animal (PT573) developed weak transient binding antibodies by enzyme-linked immunosorbent assay despite lack of specific reactivity to SIV antigens by Western blotting (Fig. 5). Also, as shown in Fig. 3 and 4, a characteristic transient decline in all lymphocyte subsets was observed during this primary phase of infection, whereas no alterations were observed in mock-infected control animals (data not shown). Although most lymphocyte subsets in the infected animals subsequently rebounded to near normal limits, CD4 lymphocyte numbers of all four macaques never returned to preinoculation values. A transient CD8 lymphocytosis was observed in the two animals with persistent antigenemia (Fig. 3), and a persistent B-cell lymphocytosis (Fig. 4) was seen in one of the macaques that had seroconverted. Sequential biopsies of peripheral lymph nodes from the animals which seroconverted demonstrated progressive follicular and paracortical hyperplasia at 4 and 16

TABLE 2. Virological and pathologic features of SIVsmE543-3-infected rapid-progressor macaques

Macaque	Histopathologic findings ^a	ISH for SIV RNA ^b
PT573	Mesenteric node, spleen: severe depletion, MNGC Peripheral nodes: severe depletion Thymus: severe depletion Gastrointestinal tract: generalized lymphohistiocytic enteritis and gastritis Meningoencephalomyelitis with diffuse gliosis, perivascular cuffs, and occasional MNGC Lung: WNL Kidney: WNL Liver: WNL	4 MNGC in sinusoids, L, M (Fig. 6A) 2 M, L in paracortical remnants 2 M, L 4 MNGC, M in GALT (Fig. 6B) 1 M, L in lamina propria 4 perivascular cuffs, glial cells, MNGC (Fig. 6C) 2 M in meninges 1 alveolar macrophages, 2 L, M in BALT 1 interstitium 0
PT583	Severe generalized lymphoid depletion with MNGC in all lymph nodes Spleen: lymphoid depletion, malarial pigment in macrophages, MNGC, no follicles Gastrointestinal tract: enteritis, gastritis, villus blunting, with lymphoid depletion in GALT Meningoencephalomyelitis with perivascular cuffs and diffuse gliosis, occasional MNGC Lung: Diffuse, severe interstitial pneumonia and alveolitis with MNGC, focal bacterial pneumonia Kidney: WNL Liver: periportal lymphoid infiltrates, malarial pigment in macrophages	4 MNGC in sinusoids; M, L paracortex (Fig. 7A) 4 MNGC, M in PALS, red pulp 4 L, M, MNGC in GALT (Fig. 6A) 3 M, L in lamina propria 3 perivascular cuffs, M, glial cells, pericytes 2 meninges, MNGC 4 MNGC, M (Fig. 7C) 2 M, L in interstitium, glomeruli (Fig. 7B) 1 M, L periportal, sinusoids

^a Only significant histopathologic findings noted. WNL, within normal limits.

^b Numbers of SIV-expressing cells detected by ISH are expressed semiquantitatively from 0 to 4, where 0 = no positive cells, 1 = 0 to 1 positive cell per high-power field (hpf), 2 = 1 to 5 cells per hpf, 3 = 10 to 50 cells per hpf, and 4 = >50 cells per hpf. Where tissues are shown in figures, the figure number is indicated in parentheses. M, macrophage; L, lymphocyte; GALT, gut-associated lymphoid tissue; BALT, bronchus-associated lymphoid tissue; PALS, periarteriolar sheath.



weeks postinoculation. In contrast, follicle formation was markedly reduced or absent in lymph nodes sampled from animals without SIV-specific antibody.

The two macaques which remained persistently antigenemic deteriorated rapidly in clinical condition and required euthanasia by 16 and 33 weeks postinoculation (Table 2). The first animal to be euthanized was severely anemic due to massive malaria (due to *Plasmodium inui*) reactivation. Although the malaria infection was successfully treated, the animal subsequently developed pneumonia, which made euthanasia necessary. Although there was no evidence of concurrent malaria infection, the remaining study animals were treated prophylactically for malaria infection. Both PT573 and PT583 exhibited high viral antigen levels in the plasma at the time of euthanasia (Fig. 3). As shown in Table 2, histopathologic lesions in tissues of these two rapid progressors were similar. Both animals exhibited severe lymphoid depletion within the paracortical regions of lymph nodes and almost complete absence of follicle formation; those rare follicles noted were small and hyalinized. Numerous multinucleated giant cells (MNGC) were observed within lymphoid tissues (particularly in sinusoidal spaces). These cells were of macrophage origin, based on positive immunostaining with a macrophage-specific monoclonal antibody, Ham56 (Dako, Inc., Copenhagen, Denmark) (data not shown). SIV-induced meningoencephalitis and myelitis with giant cells, perivascular cuffing, and diffuse gliosis were also prominent features, similar to reports of SIVmac-induced brain lesions (19, 60, 67) and findings in macaques inoculated with uncloned SIVsm isolates (7, 33, 36, 44, 65, 68). MNGC were also observed within the lung parenchyma, although the accumulations of these cells was significantly more pronounced in PT583, with obliteration of alveolar spaces throughout the lung.

The two animals which developed an SIV-specific antibody response survived longer but still became immunodeficient. One of the animals, PT580, became antigenemic again at 1 year (Fig. 4) and concurrently exhibited severe anemia (without evidence of *P. inui*), chronic diarrhea, and weight loss; this animal was euthanized at 55 weeks postinoculation. Severe, generalized lymphoid depletion was observed, with evidence of hyalinization and dissolution of germinal centers in lymph nodes and spleen. Other histopathologic abnormalities included nonbacterial valvular endocarditis, parasitic rhinitis, generalized lymphoplasmacytic enteritis, and oral candidiasis. The other animal, PT582, had evidence of immunodeficiency, based on severe peripheral CD4 lymphocyte depletion (as low as 41 cells/ μ l), lymphoid depletion in a peripheral lymph node biopsy, intermittent chronic diarrhea, and failure to thrive; these combined symptoms required euthanasia at 73 weeks postinoculation. Based on this small study, the SIVsmE543-3 cloned virus appeared to be highly pathogenic, reproducing many of the clinical and pathologic features observed in animals inoculated with uncloned SIVsmE543 (34, 36) or SIV/B670 (68).

High levels of SIV expression in tissues of rapid progressors. Both PT573 and PT583 exhibited severe generalized lymphoid depletion, almost complete absence of secondary follicles, and massive virus expression as assessed by ISH for SIV

RNA (Fig. 6 and 7). Many of the SIV RNA-expressing cells were MNGC located both within sinusoidal spaces and within paracortical regions. Virus expression was also prominent within the gastrointestinal tract, localized to macrophages and MNGC within the gastrointestinal tract-associated lymphoid tissues (Fig. 6B) but also within the lamina propria. ISH in brain sections revealed SIV expression in perivascular infiltrates and within giant cells in the meninges and multifocal granulomas, as well as a diffuse pattern of SIV-expressing glial cells scattered throughout the brain (Fig. 6C). SIV-expressing cells were observed within the lung parenchyma of both animals; generalized interstitial pneumonia with many MNGC was observed in the lungs of one animal (PT583 [Fig. 7A]). SIV-expressing macrophages within glomeruli and interstitium of the kidneys of PT583 (Fig. 7B) were an unusual finding and are a demonstration of the multisystemic dissemination of SIV in these macaques. Additionally, SIV-expressing MNGC were observed within the lamina propria of the uterus and vagina and within the corpus luteum of the ovary (not shown). In contrast to the extremely high level of virus expression observed in the antibody-negative rapid progressors, moderate numbers of ISH-positive cells were observed in tissues of PT580 (data not shown), a macaque which seroconverted and survived for approximately 1 year postinoculation. Additionally, SIV-expressing cells were primarily restricted to lymphoid tissues; no SIV expression was observed in brain, lung, liver, or kidney sections.

Neutralizing antibody response to SIVsmE543-3 infection.

The relative sensitivity of SIVsmE543-3 to neutralization in vitro was characterized with a panel of SIV-infected macaque sera that broadly neutralize other SIV variants. This panel consisted of sera from three SIVmac251-infected, long-term-surviving macaques and one macaque infected with SIVsmB670 for approximately 1 year. All samples had broadly neutralizing antibodies, as indicated by their ability to neutralize the genetically diverse variants SIVsmB670, SIVmac251, and SIVsmH4 (Table 3), which differ by as much as 20% in envelope sequences (35). Titers of neutralizing antibodies ranged from 1:763 to >1:32,805. In contrast, no neutralization of SIVsmE543-3 was detected with any of these otherwise broadly cross-neutralizing sera (Table 3).

The production of neutralizing antibodies in plasma samples from macaques infected with SIVsmE543-3 was monitored. Plasma samples from the two animals (PT573 and PT583) which failed to seroconvert had little or no neutralizing antibody to any SIV variant tested. Plasma samples from the remaining two animals (PT580 and PT582), which seroconverted, were able to neutralize SIVsmE543-3 grown in CEMx174 cells or rhesus PBMC with similar sensitivities to neutralization. Neutralizing antibody titers measured with other SIV variants were as much as 100-fold higher than those observed with SIVsmE543-3.

DISCUSSION

An infectious molecular clone of SIV, designated SIVsmE543-3, was derived from a highly pathogenic, terminal

FIG. 6. ISH for SIV RNA expression in paraffin-embedded sections from PT573, an SIVsmE543-3-infected macaque. Sections were hybridized with a digoxigenin-labeled antisense SIV probe, detected with anti-digoxigenin-alkaline phosphatase conjugate and NBT-BCIP substrate, and counterstained with nuclear fast red. (A) Mesenteric lymph node showing architectural disorganization, loss of germinal centers, depletion within the paracortical area, and large numbers of SIV-expressing cells (both multinucleated and single) (magnification, $\times 75$). (B) SIV-expressing cells within gut-associated lymphoid tissue at the ileocecal junction (magnification, $\times 75$). This pattern of expression was representative of that observed from stomach to colon. Rare positive cells were also observed within the lamina propria but were considerably more numerous in the colon. (C) Brain section at the level of the pons at low magnification, showing numerous scattered SIV-expressing cells, as well as multifocal granulomas, and perivascular cuffs of SIV-expressing cells (magnification, $\times 50$).

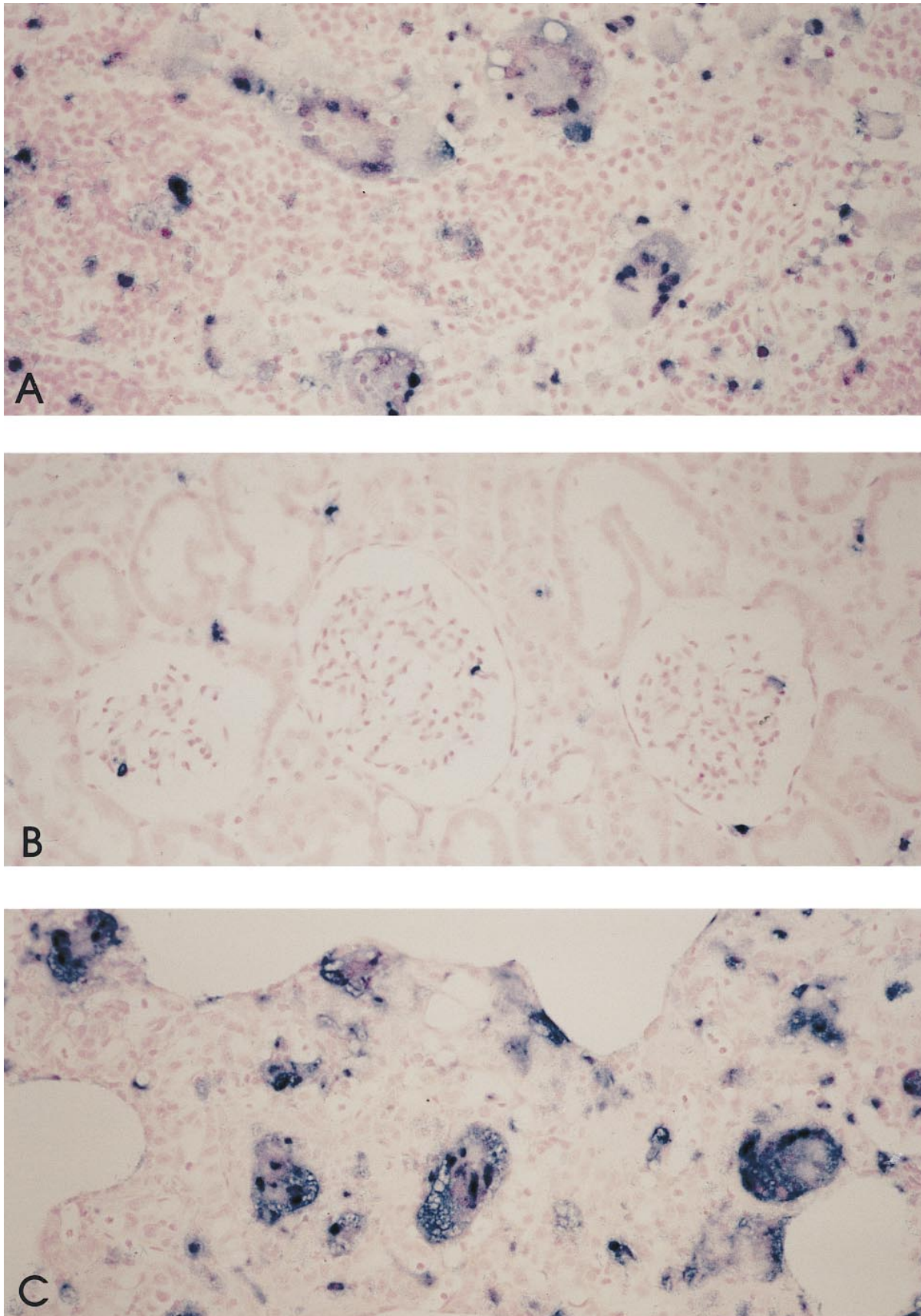


FIG. 7. ISH for SIV RNA expression in paraffin-embedded sections from PT583, an SIVsmE543-3-infected macaque. (A) Numerous MNGC, lymphocytes, and macrophages expressing SIV RNA within the mesenteric lymph node (magnification, $\times 310$). (B) SIV-expressing cells within glomeruli and interstitium of the kidney (magnification, $\times 250$). This diffuse pattern of SIV expression was observed throughout the cortical region of the kidney of this macaque. (C) Diffuse infiltration of multinucleated giant cells within the lung, demonstrating both cytoplasmic and nuclear localization of SIV mRNA with obliteration of alveolar spaces (magnification, $\times 250$).

TABLE 3. Neutralization phenotype of SIVsmE543-3 relative to other SIV isolates

Data on macaque plasma donors			Reciprocal neutralization titer with indicated cell type and SIV strains ^a					
Virus inoculum	Animal no.	Time (yr) postinfection	H9			CEMx174, 543-3	PBMC, 543-3	H9, 543-3
			smB670	mac251	smH4			
None	DU-1	NA ^b	<25	<25	<25	<25	<25	<25
SIVmac251	144-86	>4	12,207	2,294	1,046	<25	<45	<25
	145-86	>4	12,767	763	1,288	<25	<45	<25
	244-86	>4	>32,805	7,576	1,500	<25	<45	<25
SIVsmB670	T228	~1	5,832	9,045	9,718	<25	<45	<25

^a Determined with CEMx174 cells as the target cell line as described in Materials and Methods.

^b NA, not applicable.

SIV isolated from PBMC of an immunodeficient rhesus macaque (E543 [9, 34]). This molecular clone represents the second highly pathogenic, AIDS-inducing molecularly cloned virus from the SIVsm/SIVmac family, the first being the highly characterized SIVmac239 (39). SIVsmE543-3 was uniformly and highly pathogenic for macaques; three of four inoculated animals have died by 1 year postinoculation, and the remaining animal (PT582) shows clear evidence of immunosuppression (failure to thrive and CD4 count of <50 per μ l). While the pathogenicity of the E543-3 molecular clone is not unique, it possesses some novel features which make it a useful model for AIDS pathogenesis, such as (i) dual tropism for primary macaque macrophages and lymphocytes, (ii) early SIV-induced neuropathology, (iii) induction of SIV-antibody-negative, rapid progression in macaques, and (iv) relative resistance to neutralization in vitro.

SIVsmE543-3 is dual tropic and induces early encephalitis in vivo. SIVsmE543-3 was tropic for both macrophages and lymphocytes in vitro, and infection of macaques resulted in neurologic and pulmonary pathology as early as 4 months postinoculation. A predominant pathologic feature of 543-3 infection of macaques was the presence of SIV-expressing MNGC within the brain, lung, and lymphoid tissues, similar to lesions in macaques infected with uncloned SIVsm strains such as SIV/B670 (7, 44, 68) and SIVsmE660 (34) and the uncloned cell-associated SIVsmE543 virus isolate (34, 36). Based on morphology and immunohistochemistry, macrophages and macrophage-derived multinucleated cells appeared to be the predominant cell types expressing SIV. As also reported with SIV/B670, SIV-induced encephalitis occurred in those macaques which lacked measurable SIV-specific antibody (68). The properties of virus isolated from animals with neurologic disease have not been evaluated, but based on the rapidity of development and extent of brain lesions, this cloned virus appeared to be directly capable of inducing neuropathology. This capacity to induce early SIV-induced neurologic disease is a property that distinguishes it from SIVmac239 (49, 57, 60), which is highly lymphocytotropic in vitro (1, 49). Encephalitis has been observed following SIVmac239 infection, after animal passage or late in disease course, associated with the evolution of macrophage-tropic strains (19, 49, 59).

A molecularly cloned virus such as SIVsmE543 with dual tropism will be a useful adjunct to ongoing studies of SIV-induced neurovirulence using SIVmac239 derivatives (1, 49). The predominance of macrophage-tropic variants early after infection in HIV-1-infected individuals (56, 64), as well as the potential role of this cell type in mucosal transmission (61), makes the evaluation of such pathogenic, macrophage-tropic SIV variants highly relevant for both pathogenesis and vaccine studies.

Lack of SIV-specific antibody in SIVsmE543-3-infected rapid progressors. Another novel feature of macaque infection with SIVsmE543-3 was the induction of rapid progression in a high proportion of inoculated macaques. Two of four macaques inoculated with SIVsmE543-3 developed escalating viremia in the absence of a measurable SIV-specific antibody response by Western blot or neutralization assays. Whereas well-developed germinal centers were observed by 4 weeks postinfection in biopsies obtained from animals which developed SIV-specific antibodies, follicles were essentially absent from lymph node biopsies obtained from these seronegative animals. These latter animals progressed rapidly through the disease course without the intervening stages of follicular hyperplasia, and subsequent follicular dissolution and hyalinization, commonly observed in the majority of SIV-infected animals. Failure to develop germinal centers and lack of an SIV-specific antibody response are consistent with failure of these animals to mount a humoral immune response. The rapid nature of the disease course in these animals provides strong evidence for a beneficial role of humoral immunity in partial containment of SIV infection. Cell-mediated immune responses were not evaluated in this study; thus, it is possible that these animals had a cell-mediated immune response, as suggested by a transient CD8 lymphocytosis (Fig. 3).

The phenomenon of high viremia without a humoral immune response has been found previously in animals inoculated with uncloned SIV containing a complex quasispecies of viruses (24, 68). When one is using an uncloned virus, the relative contributions of virus variants and host factors are difficult if not impossible to dissect. In this study, the animals were inoculated with molecularly cloned virus, eliminating the contribution of different viral variants present during primary viremia to the subsequent disease course. Since lack of seroconversion appeared to be determined within the primary phase of infection, the contribution of evolving variants is minimized if not eliminated from consideration. The strikingly different disease courses exhibited among these four animals inoculated with a common molecularly cloned virus suggest that host factors play a major role in determining disease outcome.

Potential host factors such as immune activation at the time of infection, concurrent infections, or even the major histocompatibility complex haplotype of the animal could contribute to variability in disease outcome. For example, it is clear that immune activation, such as that observed following immunization, transiently elevates virus load potentially by providing activated lymphocytes capable of propagating SIV (or HIV) (62, 66). In the present study, one of the two rapid progressors developed fulminant reactivation of malaria (due to *P. inui*) a week prior to death, consistent with asymptomatic infection at

the time of inoculation. Preexisting malarial infection could have been a potentiating cofactor for this particular animal. However, malaria reactivation in SIV-infected macaques is not exclusively observed in rapid progressors (unpublished data) and may be simply an indicator of immunosuppression. Other opportunistic agents generally observed in macaques with AIDS were not observed, despite evidence of severe lymphoid depletion. A similar paucity of opportunistic infections was observed previously in rapid progressors infected with uncloned SIVsmE543 or SIVsmE660 (34).

The rapidity of disease onset, the lack of opportunistic infections, and the high level of viral expression are consistent with primary SIV-induced illness. The pathogenic mechanism underlying the formation and accumulation of MNGC, observed in tissues of both of these SIVsmE543-3-infected rapid progressors, is unclear. Such MNGC have been observed previously in SIV infection, primarily within the brain and lung and more infrequently within lymphoid tissues (7, 33, 60, 65, 67). MNGC are characteristic of granulomatous inflammation such as that associated with infection by intracellular bacteria such as *Mycobacterium* sp.; however, epithelioid macrophages, which are pathognomonic for granulomatous inflammation, were not observed in these SIV-infected macaques. Nevertheless, the similarities to lesions observed in granulomatous inflammation suggest that these syndromes share common pathogenic mechanisms.

Neutralizing phenotype of SIVsmE543-3. SIVsmE543-3 was highly resistant to neutralization by sera with otherwise broad and high-titer neutralizing antibodies to other related SIV variants. The difficulty in neutralizing SIVsmE543-3 is unlikely to be an epigenetic phenomenon, such as would be produced by differences in glycoprotein processing (55) or host cell proteins on the virus envelope (2, 50), since SIVsmE543-3 was equally difficult to neutralize when grown in H9 cells, CEMx174 cells, or rhesus PBMC. This resistant phenotype could be due to either the absence of specific neutralization epitopes found on other neutralization-sensitive variants of SIV or shielding of epitopes from antibody recognition. Although little is known about the heterogeneity of neutralization epitopes on different strains of SIV, minor sequence variation in the SIV gp120 can have a major influence on antibody-mediated neutralization of the virus (13). Sequence variation might explain why an international collaboration for studies with monoclonal antibodies found that SIVmac251 and SIVsmH4 share some but not all neutralization epitopes (22). Our results show that SIV strains mac251, B670, and smH4 share common neutralization epitopes and that infection with SIVsmE543-3 induces antibodies which neutralize these divergent SIV variants better than the infecting stock. A possible explanation for this latter phenomenon is that SIVsmE543-3 contains immunogenic neutralization epitopes that are poorly exposed on the native virus. Interestingly, the cryptic nature of epitopes in the V3 loop of HIV-1 gp120 was shown to protect primary monocytotropic HIV-1 from antibody neutralization *in vitro* (9), and a similar phenomenon has been observed for neutralization epitopes in the CD4 binding region of gp120 as well (63). As for HIV-1, our results suggest that sequence variation can influence the molecular environment of neutralization epitopes in the SIV envelope glycoproteins in a way that does not eliminate the epitope but rather causes different levels of epitope exposure to antibody.

While we cannot be certain about the mechanism(s) by which SIVsmE543-3 resists antibody-mediated neutralization, it is noteworthy that primary isolates of HIV-1 are similarly difficult to neutralize compared to laboratory-adapted strains of the virus (47, 48). The inability to induce antibodies that

neutralize primary HIV-1 isolates is considered an obstacle to successful HIV-1 vaccine development (28, 30). It is tempting to speculate that studies with SIVsmE543-3 in rhesus monkeys will provide valuable insights. In this regard, the identification of two molecularly cloned variants of SIV, one difficult to neutralize (i.e., SIVsmE543-3) and the other highly sensitive to neutralization (i.e., SIVsmH4), provides an opportunity to identify the molecular determinants of SIV neutralization. These efforts should improve the SIV/macaque model for studies aimed at developing a successful HIV-1 vaccine strategy.

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