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Lymphocyte-Tropic Simian Immunodeficiency Virus Causes Persistent Infection in the Brains of Rhesus Monkeys

EDWARD B. STEPHENS,*[,1] ZHEN QIAN LIU,* GE WU ZHU,* ISTVAN ADANY,* SANJAY V. JOAG,* LARRY FORESMAN,* NANCY E. J. BERMAN,† and OPENDRA NARAYAN*

*Department of Microbiology, Molecular Genetics and Immunology, Marion Merrell Dow Laboratory for Viral Pathogenesis, and †Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7240

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Molecularly cloned $SIV_{mac}239$ is the prototypical SIV_{mac} lymphocyte-tropic virus that replicates productively in lymphocytes but poorly in macrophages. In macaques, the virus causes activation and productive infection of T lymphocytes which invade the central nervous system (CNS) early after infection in the animal. However, infected animals develop immunosuppression and AIDS but rarely overt neurological disease. In this study, we examined multiple regions of the brain and spinal cord for the presence of SIV *env* sequences and histological lesions in five macaques that had been infected with $SIV_{mac}239$ for 1.7 to 2.25 years. Histopathological examination of the brain revealed no lesions consistent with encephalitis; however, viral DNA was found in all five brains. In one animal the virus caused infection in a widely disseminated pattern from the frontal cortex to the distal end of the spinal cord, whereas in the other four animals infection in the CNS occurred in a nonspecific, focal pattern. Sequence analyses were performed on gp120 sequences isolated from selected regions of the CNS and compared to gp120 sequences isolated from corresponding lymph nodes, a tissue known to support productive replication of $SIV_{mac}239$. Examination of the viral sequences from the CNS tissue from two animals (macaques 10F and 14F) revealed a low mutation rate when compared to the sequences isolated from the lymph node tissues. The percentage change in the amino acid sequence was approximately 1% for CNS clones versus $\geqslant$3% for clones isolated from the lymph node. The majority of the CNS viral sequences of macaques 10F and 14F had none of the genetic markers shown in a previous study to be associated with macrophage-tropic variants and indeed retained a nucleotide sequence of similar to the original lymphocyte-tropic virus used for inoculation despite almost 2 years of persistent infection in the animals. Construction of chimeric viruses with V1–V5 regions of selected macaque 10F and macaque 14F CNS-gp120 clones confirmed the predicted lymphocyte-tropic nature of these *env* genes. In contrast, the gp120 sequences isolated from the CNS tissue of one of the other three animals (macaque 13F) had a mutation rate comparable to that observed for the lymph node clones. The CNS clones from this animal had amino acid substitutions that were previously shown to be associated with macrophage tropism. Compared to the chimeric viruses constructed with V1–V5 sequences from macaques 10F and 14F, viruses constructed with the V1–V5 sequences of several macaque 13F brain clones did not yield infectious virus. These data suggest that following entry into the CSF early during infection in the animals, $SIV_{mac}239$ caused infection in the CNS. In some animals, the viral *env* sequences recovered by the PCR suggested that minimal replication had occurred, whereas in another macaque virus replication had progressed with gradual selection of a more macrophage-tropic genotype.

INTRODUCTION

Human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) are the causative agents of acquired immunodeficiency syndrome (AIDS). HIV-1 infection results in a gradual loss of $CD4^+$ T lymphocytes, progressive loss of immunological competence, and increased incidence of acquired infections and/or tumors. In addition to profound immunosuppression, HIV-1 causes other specific systemic complications including encephalopathy (Epstein and Gendelman, 1993; McArthur, 1987; Price and Brew, 1988; Spencer and Price, 1992), nephropathy (Bourgoigne, 1990; Cohen and Nast, 1988; Rao, 1991), and lymphocytic interstitial pneumonia (Anderson and Lee, 1988; Plata *et al.,* 1987; Rubenstien *et al.,* 1986; Travis *et al.,* 1992). HIV-1-induced lesions in the central nervous system (CNS) vary from focal meningitis to severe inflammatory and degenerative changes (Petito *et al.,* 1986; Navia *et al.,* 1986). These histopathological changes correlate with symptomatologies affecting mood, cognitive and motor functions collectively known as the AIDS cognitive/motor complex. Virological studies have shown that viruses isolated from the brains of patients with this CNS complex are invariably dualtropic (or macrophage-tropic), capable of replicating productively in both T lymphocyte and primary cultures of macrophages (Koenig *et al.,* 1986; Liu *et al.,* 1990; Sharpless *et al.,* 1992).

SIV_{mac} causes both AIDS and CNS disease in macaques, similar to HIV-1 in humans (Desrosiers *et al.,* 1991; Kestler *et al.,* 1990; Lackner *et al.,* 1990; Sharma *et al.,* 1992b). The

[1] To whom correspondence and reprint requests should be addressed. Fax: (913) 588-5599.

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virus infects both $CD4^+$ T lymphocytes and macrophages. Further, the availability of pathogenic molecular clones to SIV_{mac} has provided the potential for examining the genetic basis not only of cell tropism but also of neurological disease caused by the virus (Anderson *et al.,* 1993; Joag *et al.,* 1995; Kestler *et al.,* 1990; Naidu *et al.,* 1988). Molecularly cloned $SIV_{mac}239$ causes massive infection and subsequent activation of $CD4^+$ T lymphocytes during the first few weeks of infection in macaques. During this period, activated infected T lymphocytes cross the blood–brain barrier and appear in the cerebrospinal fluid (CSF) as early as 2 weeks postinoculation (Lackner *et al.,* 1991, 1994; Joag *et al.,* 1994a,b; Sharma *et al.,* 1992a). Animals develop a transient meningitis that subsides following the end of the early phase of T lymphocyte activation (Lackner *et al.,* 1991, 1994; Joag *et al.,* 1994a). However, $SIV_{mac}239$ rarely causes overt SIV-induced encephalitis in macaques. In those cases in which macaques developed encephalitis following inoculation with $SIV_{mac}239$, the virus isolated from the brain was dualtropic with the ability to replicate in macrophages at a higher level than the original $SIV_{mac}239$, suggesting that the selection of dualtropic variants is a prerequisite for development of SIV-induced encephalitis (Desrosiers *et al.,* 1991; Sharma *et al.,* 1992b; Anderson *et al.,* 1993). Thus, while the current data indicate that $SIV_{mac}239$ can be found in the brain early during infection, the question remains whether this virus could establish and maintain infection of the neuropil without prior mutation to a macrophage-tropic virus (i.e., is this lymphocyte-tropic virus neurotropic?). We examined the CNS tissues from five $SIV_{mac}239$-infected animals euthanized 1.7 to 2.25 years after inoculation. None of the brains had histological evidence of encephalitis. Infectious virus was not recovered from any brain samples. However, we identified SIV_{mac} sequences in different regions of the CNS of infected animals using the PCR technique. Nucleotide sequences of the viral gp120s obtained from selected regions of the CNS and the lymph node, a tissue of high virus replication, were compared to those of the inoculum virus. Sequence analyses revealed the virus *env* sequences recovered from two of three animals had a very low nucleotide substitution rate indicative of a minimal rate of replication and a lymphocyte-tropic genotype resembling the inoculum virus. The third animal had a high nucleotide substitution rate in both the brain and the lymph node but the virus evolved along divergent pathways. These results indicate that $SIV_{mac}239$ is highly neuroinvasive and causes persistent infection in the CNS. Replication in this tissue, however, may be of a slow, restricted type or rapid, similar to that occurring in the lymphoid tissue.

MATERIALS AND METHODS

Cell cultures and virus stocks

The CEMx174 cell line was used as the indicator cell line to measure infectivity of $SIV_{mac}239$. CEMx174 is a human B–T hybrid cell line (Salter *et al.,* 1985) and was maintained in RPMI 1640, supplemented with 10 m*M* HEPES buffer, pH 7.3, 5×10^{-5} β-mercaptoethanol, 2 m*M* glutamine, 50 μg/ml gentamicin, and 10% fetal bovine serum (FBS). CV-1 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 50 μg/ml of gentamicin and 10% FBS. $SIV_{mac}239$ was provided by Dr. R. C. Desrosiers at the New England Primate Center at Harvard University (Regier and Desrosiers, 1990). The lymphocyte-tropic properties of this virus have been described previously (Mori *et al.,* 1992, 1993; Stephens *et al.,* 1995). $SIV_{mac}LG$-1 is a chimeric virus in which the V1–V5 region of gp120 from $SIV_{mac}239$ was replaced with the corresponding region from a gp120 clone (LG-1) isolated from the lung tissue of macaque 7F that developed severe interstitial pneumonia 1 year after inoculation with $SIV_{mac}239$ (Zhu *et al.,* 1995). $SIV_{mac}LG$-1 replicates productively in rhesus macrophages and causes massive syncytial cytopathology within 4 to 5 days postinoculation.

Animals and virus inoculations

In an earlier study on the pathogenesis of $SIV_{mac}239$ infection of rhesus macaques, we had inoculated 11 animals with the virus. Four developed full-blown AIDS within 2 years and the others, while infected, remained healthy (Joag *et al.,* 1994b). All animals were euthanized by exsanguination under anesthesia and various tissues were collected for analyses of virus burden and pathological changes. Samples of CNS tissue collected for study included the frontal cortex, parietal cortex, midbrain, brain stem, and three segments of the spinal cord. CNS and lymphoid tissues were collected from five of the animals which were euthanized at the indicated periods: 13F, 88 weeks; 10F, 89 weeks; 14F, 92 weeks; 68, 117 weeks; 138, 116 weeks; and 197, 117 weeks. None of these macaques had developed signs of neurological disease.

Processing of blood and tissue samples

Heparinized blood was centrifuged to separate the plasma from the buffy coat. Plasma was assayed directly for infectivity in CEMx174 cells and the cells were centrifuged through Ficoll–Hypaque density gradients to isolate peripheral blood mononuclear cells (PBMC). Portions of tissue specimens obtained at necropsy were processed as follows: (1) frozen at −80°C and used as the source of genomic DNA; (2) used to prepare cell-free, 10% homogenates (w/v) which were assayed for infectivity in CEMx174 cells; (3) used to prepare single cell suspensions by passing through a fine mesh (lymph node) and the number of infected cells was assessed by both a polymerase chain reaction/infected cell assay (PCR–ICA) and cocultivation with CEMX174 cells; (4) ali-

quots of cells were cultivated in macrophage differentiation medium (MDM) and in sRPM1 containing phytohemagglutinin/IL-2 to test for infectious virus in macrophages and lymphocytes; (5) used to prepare tissue explants in MDM to test for productive virus replication in tissue macrophages; and (6) one half of the brain was fixed in 10% Zn^{2+}–Formalin for histopathological examination.

Assessment of virus burden in blood and lymph node cells

To determine the number of cells actively producing virus, infectious cell assays (ICA) were performed. Serial two-fold dilutions of PBMC and lymph node cells were inoculated onto CEMx174 cells, which were then observed for development of syncytial cytopathic effects (CPE) during a 7-day period. The results were expressed as the number of infectious cells per 10^5 cells (Joag *et al.,* 1994a).

To determine if activated T-lymphocytes would produce infectious virus, PBMCs were cultured in the presence of 1 μg/ml phytohemagglutinin (PHA-P, Wellcome) for 2 days. The cells were centrifuged, and the cell pellets were resuspended in sRPMI + 100 U/ml rhuIL-2 (Cetus) and cultured for an additional 5 days, after which they were centrifuged again and the cell-free culture medium assayed for infectivity in CEMx174 cultures (Joag *et al.,* 1994a).

Explant cultures yielded cultures of macrophages that migrated from the tissue fragments. Supernatant fluids from these cultures were inoculated into cultures of CEMx174 cells to test for cytopathic virus and the indicator cells were also added to macrophage cultures to test for the development of fusion (Sharma *et al.,* 1992a,b).

Tissue homogenates were prepared by Dounce homogenization (10% w/v), clarified by centrifugation to remove cell debris and assayed for virus infectivity in CEMx174 cells and for p27 levels.

A PCR–ICA was used to determine the number of virus-infected cells in various cell suspensions. This assay was similar to a procedure described previously (Poznansky *et al.,* 1991) with certain modifications (Joag *et al.,* 1994b). Cell suspensions were diluted to 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 cells per milliliter. Cells in the various dilutions were then lysed and digested as described previously (Poznansky *et al.,* 1991). Two rounds of PCR amplification were used to detect SIV *gag* sequences. In the first round, SIV oligonucleotide primers used were ES8, 5′-GATGGGCGTGAGAAACTCCGTCTT-3′ (sense), and ES21, 5′-CCTCCTCTGCCGCTAGATGGTGCTGTTG-3′ (antisense), which are complementary to bases 1052 to 1075 and 1423 to 1450 of the $SIV_{mac}239$ *gag* gene, respectively (Regier and Desrosiers, 1990). To standardize cell numbers, the fourth exon of β-actin was amplified with oligonucleotide primers ES30, 5′-TCATGTTTGAGACCTTCAACACCCCAG-5′ (sense), and ES32, 5′-CCAGGAAGGAAGGCTGGAAGAGTGCC-5′ (antisense), complementary to the published sequence (Nakijima-Iijima *et al.,* 1985). The PCR amplification was performed using the following conditions: Denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 3 min. To increase the sensitivity of the reaction, 1 μl of the first PCR product was used as a template for a second amplification using the same conditions. The nested SIV primers used were ES19, 5′-GTTGAAGCATGTAGTATGGGCAGC-3′ (sense), and ES20, 5′-GCCTCAGGGCAGCGGAACCGCTCA-3′ (antisense), which are complementary to bases 1142 to 1165 and 1356 to 1382 of $SIV_{mac}239$, respectively. The nested β-actin primers used were ES31, 5′-CCCCAGCCATGTACGTTGCTATCC-3′ (sense), and ES33, 5′-GCCTCAGGGCAGCGGAACCGCTCA-3′ (antisense). Samples were amplified for another 35 cycles as described above. Following the second round of amplification, a 10-μl aliquot was removed and run on a 1.5% agarose gel and bands were visualized by staining with ethidium bromide. The results were calculated as the number of infected cells/10^5 cells.

PCR amplification of gp120 sequences from neural and nonneural tissues

Total cellular genomic DNA was extracted from regions of the brain and spinal cord and nonneural tissues and used as a template in a nested polymerase chain reactions (PCR) (Saiki *et al.,* 1985, 1988) to amplify SIV gp120 sequences. The oligonucleotide primers used in the first round were ES12, 5′-GGCTAAGGCTAATACATCTTCTGCATC-3′, and ES14, 5′-ACCCAAGAACCCTAGCACAAAGACCCC-3′, which are complementary to bases 6565 to 6591 and 8179 and 8205 of $SIV_{mac}239$, respectively (Regier and Desrosiers, 1990). One microgram of genomic DNA was used in the PCR containing 4.0 m*M* $MgCl_2$, 200 μ*M* each of the four deoxynucleotide triphosphates, 100 p*M* each oligonucleotide primer, and 2.5 U of *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, CT). The template was denatured at 95°C for 3 min and PCR amplification was performed with an automated DNA Thermal Cycler (Perkin–Elmer Cetus) for 35 cycles using the following profile: denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 3 min. Amplification was completed by incubation of the PCR for 10 min at 72°C. One microliter of the PCR product from above was used in a nested PCR using the same reaction conditions described above. For the second round of amplification, the nested set of primers were ES11, 5′-GTAAGTATGGGATGTCTTGGGAATCAG-3′, and ES13, 5′-GACCCCTCTTTTATTTCTTGAGGTGCC-3′, which are complementary to bases 6598 to 6624 and 8158 to 8184 of the $SIV_{mac}239$ genome, respectively. To

confirm the specificity of the PCR products, the DNA in the gel was transferred onto nitrocellulose by the Southern technique and then hybridized with a ^{32}P-labeled gp120 probe generated by a random primer labeling method (Feinberg and Vogelstein, 1983; Vogelstein and Feinberg, 1984). The blot was washed for 30 min at 65°C with 2× SSC/0.5% SDS, 0.2× SSC/0.5% SDS, and finally with 0.1× SSC/0.5% SDS. The blot was then exposed to X-Omat film and processed using standard autoradiographic procedures.

Molecular cloning of amplified DNA products and sequence analyses

The amplified gp120 gene products were electrophoresed in a 1% agarose gel, electroeluted, and then molecularly cloned into the *Sma*I site of the pGEM7Zf(−) vector as described previously (Zhu *et al.,* 1995). Plasmids containing gp120 sequences from three separate PCR amplifications were sequenced by primer directed dideoxy sequencing using Sequenase enzyme (U.S. Biochemicals, Cleveland, OH) and [^{35}S]dATP. SIV gp120 sequences were compared to the original $SIV_{mac}239$ sequence using PCGENE sequence analysis software programs. For comparative purposes, the designation of the variable regions (V1–V5) of SIV gp120 were based on results published previously (Kodama *et al.,* 1993).

Construction of chimeric viruses

To determine if the gp120 sequences isolated from the CNS would confer macrophage tropism onto the lymphocyte-tropic $SIV_{mac}239$, we constructed chimeric viruses in which the majority of the $SIV_{mac}239$ gp120 (amino acids 107–490) were replaced with the corresponding region from gp120 sequences from macaques 10F, 14F, and 13F. The $SIV_{mac}239$ genome in plasmid pBS was digested with *Nsi*I and *Cla*I to release a 1148-base-pair fragment encoding the V1 to V5 regions of gp120. The plasmid was gel purified and ligated with the *Nsi*I/*Cla*I fragment isolated from the pGEM3Zf(−) vector containing the appropriate V1–V5 sequences. The resulting ligated DNA was used to transform JM109 bacteria and the resulting transformants were screened for the presence of a *Nsi*I/*Cla*I insert. To ensure that the insert corresponded to the appropriate sequence and that no premature termination codons were present in the DNA, the entire gp120 region was sequenced. Plasmids with the correct *Nsi*I/*Cla*I fragments were used to transfect CEMx174 cells. Syncytial CPE was observed within 48 hr of transfection and increased with continued incubation of cultures. Stock viruses were prepared at 7 days posttransfection and titrated in CEMx174 cells.

Assessment of macrophage tropism of chimeric viruses

The macrophage-tropic nature of the various virus constructs was assessed using the criteria established for the lymphocyte-tropic $SIV_{mac}239$ and a macrophage-tropic $SIV_{mac}239/17E$ as reported previously (Stephens *et al.,* 1995; Zhu *et al.,* 1995a). Briefly, these studies showed that $SIV_{mac}239$ caused infection in rhesus macrophages but virus protein biosynthesis in these cells was greatly reduced when compared to the macrophage-tropic $SIV_{mac}239/17E$. Immunoprecipitation studies showed that only minimal amounts of gp120 are released from $SIV_{mac}239$-infected macrophages whereas the viral glycoprotein (gp120) was efficiently released from $SIV_{mac}239/17E$-infected macrophages. Thus, technically, both viruses are macrophage-tropic. In this report we assign the term macrophage-tropic to those viruses which, during infection in macrophages, efficiently released radiolabeled gp120 into the culture medium. Monolayers of macrophage cultures in 35-mm dishes were washed three times with RPMI 1640, inoculated with 0.1 ml of undiluted virus stocks in 0.5 ml of MDM, incubated for 2 hr at 37°C, and then supplemented with 2 ml of fresh MDM and reincubated for 6 days. Cells were then incubated in methionine/cysteine-free medium for 2 hr and then radiolabeled with 200 μCi/ml of [^{35}S]-methionine/cysteine (ICN, Costa Mesa, CA) for 16–18 hr. The culture medium was harvested and cell lysates were prepared as described previously and processed for immunoprecipitation analyses (Stephens *et al.,* 1991, 1992, 1995). Culture medium and cell lysates were made 1× with respect to RIPA buffer (50 m*M* Tris–HCl, pH 7.5, 50 m*M* NaCl, 0.5% deoxycholate, 0.2% SDS, and 10 m*M* EDTA) and SIV-specific proteins immunoprecipitated using 10 μl of a SIV-positive plasma and protein A–Sepharose as described previously (Stephens *et al.,* 1995). Immunoprecipitates on beads were washed three times with 1× RIPA buffer and the bound proteins boiled 5 min in 50 μl of 2× SDS sample reducing buffer (Laemmli, 1970). Immunoprecipitated proteins were separated by SDS–polyacrylamide gel electrophoresis (8.5% gel) and proteins were visualized using standard autoradiographic techniques.

In addition to immunoprecipitation analyses, infected macrophage cultures were stained for the presence of p27 antigen to assess the extent of infection. Macrophage cultures were established on coverslips in 35-mm plates as described previously (Stephens *et al.,* 1995). For controls, macrophage cultures were inoculated with either $SIV_{mac}239$ or $SIV_{mac}LG-1$ or remained uninfected. At 5 days postinfection, cultures were washed once with sRPMI and fixed in a solution of Zn^{2+}–Formalin. Cells on coverslips were pretreated with 0.3% H_2O_2/0.1% NaN_3 for 30 min to remove endogenous peroxidase activity, washed with PBS, and then blocked using 5% goat serum for 60 min at room temperature. Cells were reacted with a mouse monoclonal antibody directed against the p27 core antigen (FA-2) overnight at room temperature. Cells were washed three times with PBS/0.1% Triton X-100,

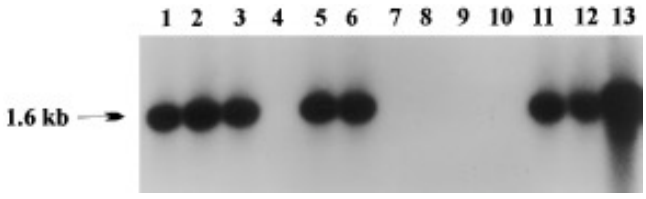


FIG. 1. Detection of SIV_{mac}gp120 sequences in non-CNS and CNS tissues of macaque 14F. DNA was isolated from CNS and non-CNS tissues of macaque 14F and gp120 sequences amplified using nested PCR as described under Materials and Methods. Aliquots from each reaction were run on a 1.0% agarose gel, the DNA was transferred onto nitrocellulose, and the membranes were hybridized with a ^{32}P-labeled derived from the SIV_{mac}239 *env* gene. Membranes were washed and exposed to X-OMAT film. Lane 1, spleen; Lane 2, lymph node; Lane 3, lung; Lane 4, liver; Lane 5, kidney; Lane 6, intestine; Lane 7, frontal cortex; Lane 8, parietal cortex; Lane 9, midbrain; Lane 10, spinal ganglia; Lane 11, brain stem; Lane 12, cervical spinal cord; Lane 13, positive control.

reacted with a biotinylated goat anti-mouse antibody for 30 min, and washed again with PBS/0.1% Triton X-100. Cells were reacted with ABC–horseradish peroxidase for 30 min at room temperature and then reacted with DAB substrate. Cells were counterstained with hematoxylin, dehydrated, and then mounted onto glass slides.

p27 assays

Cell-free homogenates were prepared from infected and uninfected regions of the CNS from animals were inoculated with SIV_{mac}239 (as determined by the presence or absence of SIVmac *gag* gene in nested PCR as described above). Protein concentrations were determined and samples with equivalent amounts of proteins were analyzed for the levels of p27 core antigen in homogenates (samples were adjusted to equivalent protein concentrations) determined using antigen capture assays (Coulter Corp., Hialeah, FL).

RESULTS

Virus burden in macaques 10F, 13F, 14F, 138, and 197

None of the animals examined in this study had simian AIDS at the time of necropsy which varied from 88 to 117 weeks postinoculation. Virus replication was detected in all visceral organs except the liver, as exemplified by examination of tissues from macaque 14F (Fig. 1). The virus burden in the PBMC, lymph node, and brain tissues was evaluated by several procedures and the data are summarized in Table 1. Homogenates of brain tissue were uniformly negative for virus infectivity. In addition, explant cultures from the brain were negative for virus infectivity after coculturing with CEMx174 cells. In contrast, homogenates prepared from the lymph nodes three macaques (macaques 10F, 14F, and 138) yielded virus infectivity. Supernatant fluids of PHA/IL-2 cultures from PBMC and lymph node cells had infectivity titers the ranged from 16 to 100,000 $TCID_{50}$ per milliliter in four of the animals analyzed, showing the presence of virus in lymphocytes. The only animal that did not yield virus following treatment of cells with PHA/IL-2 was macaque 197 which had the lowest virus burden of the five animals. Infectious centers assays revealed that macaque 10F had the highest number of infectious cells in preparations from the lymph node and PBMC (33–67 infected cells/100,000). This was confirmed by the use of a PCR–ICA which revealed that 1 of 100 cells from lymph node tissue and PBMC of macaque 10F were positive for viral sequences. From these data we conclude that lymphocyte-tropic virus was present and replicated efficiently in lymphoid tissues. Further, the inability to recover virus from explants of the brain suggested that infectious macrophage-tropic variants had not been selected in the brain. Despite this, viral DNA was found in the CNS of all five macaques examined with two patterns of virus distribution. The first pattern (macaque 10F) was characterized by a global distribution of gp120 sequences in all regions of the CNS examined. The second pattern (macaques 14F, 13F, 197, and 138) was characterized by a regional localization of gp120 sequences. In this type of CNS involvement, gp120 sequences were detected in one or more but not in all regions of the CNS. We detected gp120 sequences in two (brain stem and cervical spinal cord) of five regions of macaque 14F, one (the frontal cortex) of five regions examined from macaque 13F, one (cervical spinal cord) of five regions examined from macaque 197, and three (frontal cortex, brain stem, and cervical spinal cord) of four regions examined from macaque 138. The location of SIV_{mac} *env* sequences in the CNS for the five macaques is summarized in Table 2.

The presence of the viral genome correlates with the low levels of p27 antigen in tissues

We examined several regions of the CNS for the presence or absence of p27 antigen that were positive (macaque 14F spinal cord, 13F frontal brain, and 10F frontal brain) and negative (macaque 14F frontal brain and macaque 13F spinal cord) for SIV_{mac} sequences by PCR as described above. The results shown in Fig. 2 indicate that for macaques 10F and 14F, low levels of p27 antigen were detected in regions of the CNS that were shown to have viral sequences but not in those regions that were negative for virus sequences by the PCR. Contrasting with these results, we were unable to detect p27 antigen in samples prepared from the frontal cortex and spinal cord of macaque 13F. These results suggest that in two of the three macaques analyzed, p27 antigen was detected suggesting that a low-level, minimally productive was occurring in the brain of these animals. Antigen was not detected in the plasma derived from the three animals (data not shown) and samples prepared from the

TABLE 1

Virus Burden in Macaques 10F, 13F, 14F, 197, and 138

Animal	Period of infection (weeks)	Tissue	Titer of virus in homogenates[a]	Titer of virus from PHA/IL-2 cultures[a]	ICA[b]	PCR–ICA[c]
10F	89					
		Brain	Neg	NA[d]	NA	NA
		PBMC	NA	1.2	67	1000
		Lymph node	2.0	3.3	33	1000
13F	88					
		Brain	Neg	NA	NA	NA
		PBMC	NA	2.4	2	100
		Lymph node	Neg	2.4	<1	100
14F	92					
		Brain	Neg	NA	NA	NA
		PBMC	NA	2.8	1	100
		Lymph node	1.0	5.0	33	100
197	117					
		Brain	Neg	NA	NA	NA
		PBMC	NA	Neg	2	10
		Lymph node	Neg	Neg	<1	10
138	116					
		Brain	Neg	NA	NA	NA
		PBMC	NA	1.2	33	100
		Lymph node	1.0	2.1	16	100

[a] Expressed as $\log_{10}$ $TCID_{50}$ per milliliter.
[b] Number of infectious cells per 100,000 by cytopathology assay.
[c] Number of infected cells per 100,000 as determined by PCR–ICA.
[d] NA, Not applicable.

lymph node of macaque 10F were strongly positive for p27 antigen (Fig. 2).

Sequence analyses of gp120 clones from the CNS and lymph node

Because all five macaques in this study were positive for SIV gp120 sequences in at least one region of the CNS, we selected a positive region at random from three of the animals (frontal cortex from macaque 10F, frontal cortex from macaque 13F, and spinal cord from macaque 14F) and determined the nature of the gp120 sequences (i.e., do they have genetic markers for macrophage-tropic or lymphocyte-tropic viruses) in the CNS tissue and compared these sequences to those present in the lymph node, a tissue known to support the replication of $SIV_{mac}239$ (Kestler *et al.,* 1990; Sharma *et al.,* 1992a). The predicted protein sequences from the sequence analyses of these clones are shown in Fig. 3. Examination of the gp120 sequences isolated from the spinal cord of macaque 14F revealed a paucity of amino acid changes in four of the five clones and an overall mutation rate of 0.51%. Thus, although this animal was harboring this virus for 1.7 years, these four sequences resembled the input virus genome. These same four clones lacked of the V67M, K176E, and G382R substitutions within gp120 previously shown to be associated with macrophage tropism of $SIV_{mac}239$ variants. The same four clones all had amino acid substitutions at positions 132P and 141E indicating a tissue selection of gp120 variants in brain. The fifth clone isolated from macaque 14F spinal cord tissue had the V67M substitution but none of the other changes associated previously with macrophage tropism; it also had a higher amino acid substitution rate. In contrast to the gp120 sequences isolated from the spinal cord, sequences from the lymph node of macaque 14F had a

TABLE 2

Distribution of SIV Sequences in the CNS of $SIV_{mac}239$-Inoculated Animals

	Tissue[a]				
Animal	Frontal cortex	Parietal cortex	Midbrain	Brain stem	Cervical spinal cord
10F	(+)	(+)	ND	(+)	(+)
13F	(+)	(−)	ND	(−)	(−)
14F	(−)	(−)	(−)	(+)	(+)
138	(+)	ND	(−)	(+)	(+)
197	(−)	ND	(−)	(−)	(+)

[a] The presence of SIV sequences in tissue was determined using PCR and oligonucleotide primers that amplified the SIV_{mac} gp120 gene.

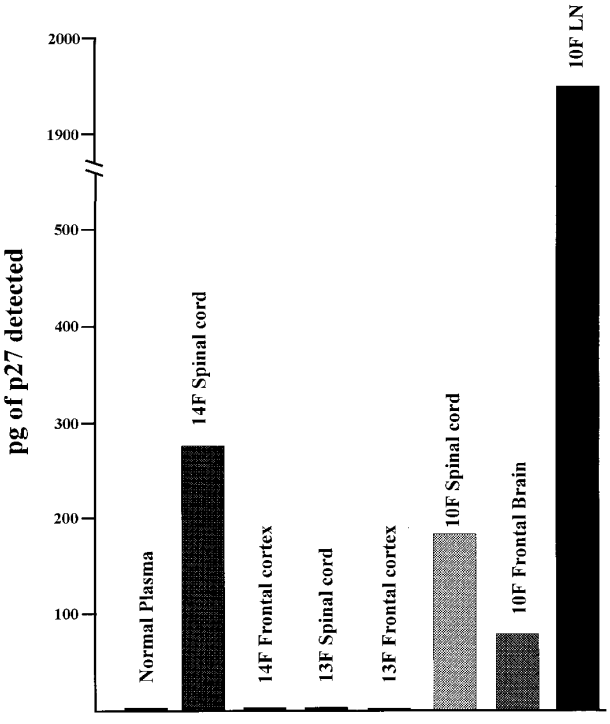


FIG. 2. Levels of p27 antigen detected in selected CNS tissues of $SIV_{mac}239$-infected macaques (10F, 13F, and 14F). Plasma from these animals were also included as negative controls and a homogenate prepared from 10F lymph node tissue (positive for infectious virus) was included as a positive control and was greater than the 1950 pg/ml.

higher overall mutation rate of 1.28% and a change in the amino acid sequence of approximately 3.0% (Fig. 3 and Table 3). The differences in the overall mutation and amino acid substitution rates between the brain and lymph node clones of macaque 14F were found to be statistically significant ($P < 0.05$). All gp120 sequences from the lymph node had a methionine or valine substitution at position 67. The gp120 clones isolated from the lymph node tissue also had amino acid substitutions that suggested tissue-specific selection of gp120s. These amino acid substitutions included a S149P, W345R, G347E, and an A416T.

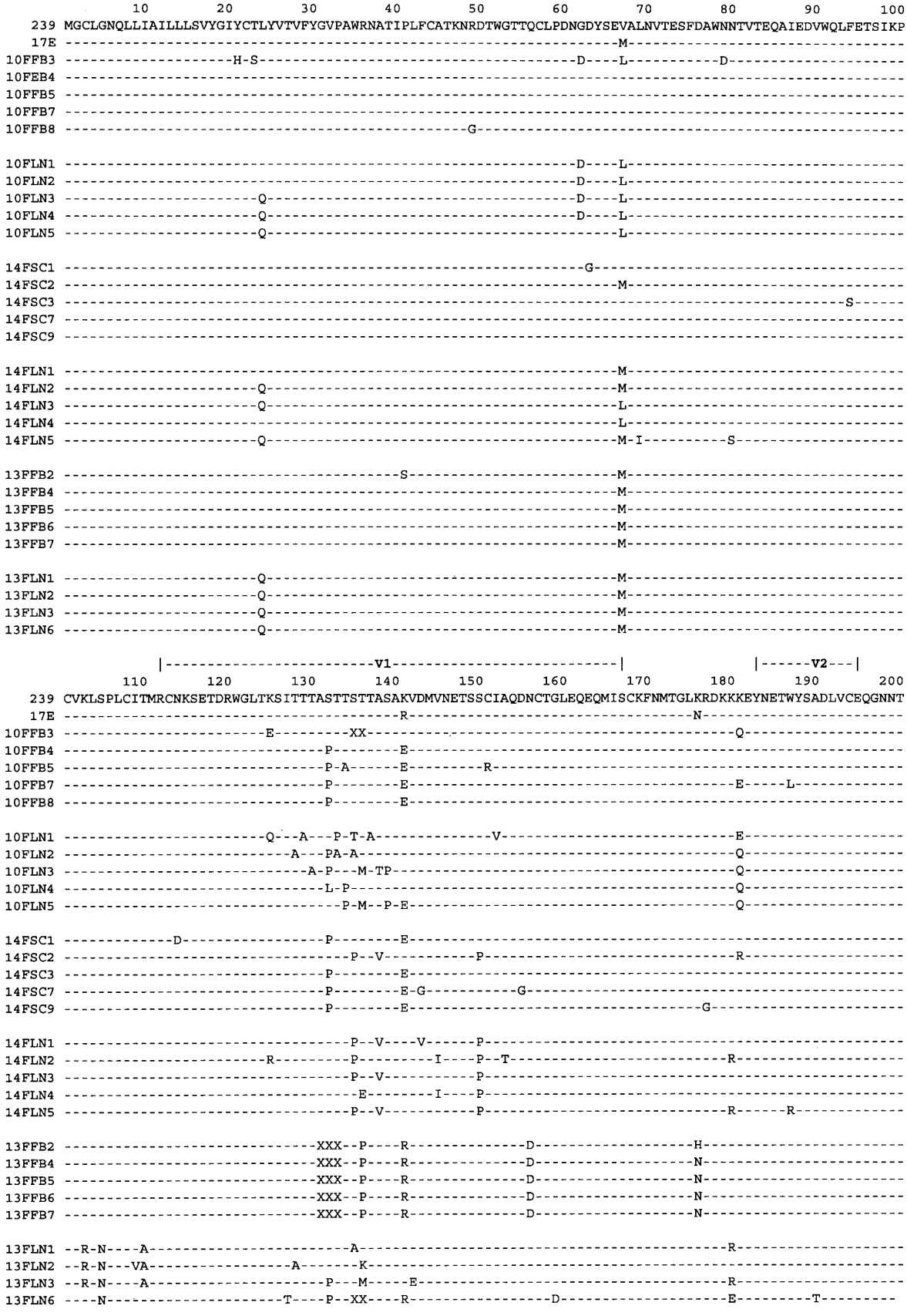
Similar to macaque 14F, analysis of gp120 clones isolated from the frontal cortex of macaque 10F revealed an overall low mutation rate of 0.54% and amino acid substitution rates of 1.18% (Fig. 3 and Table 3). Interestingly, all of the macaque 10F clones from the frontal cortex with low mutation rates also had S132P and K141E substitutions seen in the macaque 14F spinal cord clones. Examination of the macaque 10F gp120 clones isolated from the lymph node tissue revealed an average nucleotide substitution rate of 1.68% and overall mutation rate of 2.56%. All macaque 10F clones had a V67L, K180E/Q, R292N, Y304F, W345R, and K349E/N substitutions as well as four to six amino acid deletions in the V4 region, again emphasizing differences between viral sequences obtained from the brain and lymph node tissues.

Contrasting with the results from macaques 10F and 14F, the gp120 clones isolated from the brain of macaque 13F had a mutation rate that was similar to those of clones obtained from the lymph node (Table 3). The clones from the brain of this animal had an average nucleotide substitution rate of 1.46% and an overall mutation rate of 2.03%, whereas the average nucleotide substitution rate for clones from the lymph node was 1.19% and an overall mutation rate of 2.43%. While the average change in the amino acid sequence was approximately 1% for the CNS-gp120 clones from macaques 10F and 14F, the average change in the amino acid sequence was 3.7 and 4.0% for clones isolated from the frontal cortex and lymph node of macaque 13F, respectively. The clones isolated from the brain of macaque 13F had genetic markers previously shown to be associated with macrophage tropism, including V67M, K176N, and G382R amino acid substitutions (Mori *et al.,* 1992). These sequences also had other amino acid substitutions (T135P, K140R, N155D, M308I, and I323M) and deletions in the V1 region, suggesting that this virus may have undergone clonal expansion in the brain. These amino acid substitutions were not found in the gp120 clones from the lymph node tissue. We also compared the amino acid sequence from the macaque 13F brain clones with the gp120 of $SIV_{mac}239/17E$, a virus whose *env* gene was derived from the brain of a macaque that developed encephalitis during neuroadaptation of $SIV_{mac}239$ (Anderson *et al.,* 1993). Interestingly, the predicted amino acid sequence of the gp120s from the macaque 13F brain had amino acid substitutions at seven of the nine residues changed in $SIV_{mac}239/17E$ (Fig. 3). This strongly suggested that selection of certain viral genotypes was occurring in the brain.

Chimeric viruses constructed with the V1–V5 gp120 sequences isolated from 10F and 14F brain are lymphocyte-tropic viruses

Because sequence analyses suggested that the gp120 genes isolated from CNS clones isolated from macaques

FIG. 3. Predicted amino acid sequence of gp120 clones isolated from the lymph node and brain tissues from of macaques 10F, 13F, and 14F at necropsy. FB refers to the frontal cortex, SC refers to spinal cord, and LN refers to lymph node as the tissue source of DNA for PCR amplification and cloning. Shown above the sequences of the 29 clones is the sequence of $SIV_{mac}239$ clone (Regiers and Desrosiers, 1990) and the $SIV_{mac}239/17E$ clone (Anderson *et al.,* 1993; Zhu *et al.,* 1995a). Dashes (-) represent amino acid identity; crosses (x) represent deletions. For comparative purposes, brackets corresponding to the variable regions V1, V2, "V3," V4, and V5 as described previously (Mori and Desrosiers, 1993) were placed above the $SIV_{mac}239$ sequence.


10 20 30 40 50 60 70 80 90 100
239 MGCLGNQLLIAILLLSVYGIYCTLYVTVFYGVPAWRNATIPLFCATKNRDTWGTTQCLPDNGDYSEVALNVTESFDAWNNTVTEQAIEDVWQLFETSIKP
17E
10FFB3
10FEB4
10FFB5
10FFB7
10FFB8
10FLN1
10FLN2
10FLN3
10FLN4
10FLN5
14FSC1
14FSC2
14FSC3
14FSC7
14FSC9
14FLN1
14FLN2
14FLN3
14FLN4
14FLN5
13FFB2
13FFB4
13FFB5
13FFB6
13FFB7
13FLN1
13FLN2
13FLN3
13FLN6
V1
V2
110 120 130 140 150 160 170 180 190 200
239 CVKLSPLCITMRCNKSETDRWGLTKSITTTASTTSTTASAKVDMVNETSSCIAQDNCTGLEQEQMISCKFNMTGLKRDKKKEYNETWYSADLVCEQGNNT
17E
10FFB3
10FFB4
10FFB5
10FFB7
10FFB8
10FLN1
10FLN2
10FLN3
10FLN4
10FLN5
14FSC1
14FSC2
14FSC3
14FSC7
14FSC9
14FLN1
14FLN2
14FLN3
14FLN4
14FLN5
13FFB2
13FFB4
13FFB5
13FFB6
13FFB7
13FLN1
13FLN2
13FLN3
13FLN6

```
                 210        220        230        240        250        260        270        280        290        300
   239 GNESRCYMNHCNTSVIQESCDKHYWDAIRFRYCAPPGYALLRCNDTNYSGFMPKCSKVVVSSCTRMMETQTSTWFGFNGTRAENRTYIYWHGRDNRTIIS
   17E ----------------N-------------------------------------------------------------------------------------
10FFB3 ---------------------------------------------------------------------------------------------------N-------
10FFB4 ---------------------------------------------------------T-------------------------------------------------
10FFB5 ---------------------------------------------------------Y-------------------------------------------------
10FFB7 ---------------------------------------------------------------------------------------------------------
10FFB8 ---------------------------------------------------------------------------------------------------------

10FLN1 --------------I-------------------------------------------------------------------------------------N-------
10FLN2 ---------------------------------------------------------------------------------------------------N-------
10FLN3 ----------------------------------------K--N-------------I-------------------------------------------N-------
10FLN4 ---------------------------------------------------------------------------------------------------N-------
10FLN5 ----------Y----------------------------------------------------------------------------------------N-------

14FSC1 ---------------------------------------------------------------------------------------------------------
14FSC2 ---------------------------------------------------------------------------------------------------------
14FSC3 ---------------------------------------------------------------------------------------------------------
14FSC7 ---------------------------------------------------------------------------------------------------------
14FSC9 ---------------------------------------------------------------------------------------------------------

14FLN1 D---------R----------E-----------------------------------------------------------------------------------
14FLN2 ---------------------------------------------------------------------------------------------------------
14FLN3 ---------------------------------------------------------------------------------------------------------
14FLN4 ---------------------------------------------------------------------------------------------------------
14FLN5 ---------------------------------------------------------------------------------------------------------

13FFB2 -------------------------------------------------A-------------------------------------------------------
13FFB4 ---------------------------------------------------------------------------------------------------------
13FFB5 -------------------------------------------------A-------------------------------------------------------
13FFB6 ---------------------------------------------------------------------------------------------------------
13FFB7 -------------------------------------------------A-------------------------------------------------------

13FLN1 ----------------------------------------------------N----------------------------------------------------
13FLN2 ------------------------------------------------D------N----------G-----------------------------------
13FLN3 ----------------------------------------------------N----------------------------------------------------
13FLN6 D---------------------------------------------------R----------------------------------------------------

                |-------------- "V3" ------------|
                310        320        330        340        350        360        370        380        390        400
   239 LNKYYNLTMKCRRPGNKTVLPVTIMSGLVFHSQPINDRPKQAWCWFGGKWKDAIKEVKQTIVKHPRYTGTNNTDKINLTAPGGGDPEVTFMWTNCRGEFL
   17E --------I-----------------------R-----R-------------------------------------------R-----------------
10FFB3 ---F----IR-----------------------------------------------------------------------------------------
10FFB4 ---------------------------------------------------------------------------------------------------------
10FFB5 ---------------------------------------------------------------------------------------------------------
10FFB7 ----------------------------A----------------------------------------------------------------------------
10FFB8 ---------------------------------------------------------------------------------------------------------

10FLN1 ---F-----R-------------------S--------------R---N-------------------------------------------------------
10FLN2 ---F-----R---------------------------R----R---E---------------------------S----------------------------
10FLN3 ---F-----------------------------------R---E-------------------------------------------------------------
10FLN4 ---F-----R-----------------------------R----R---E-------------------------------------------------------
10FLN5 ---F-----R-----------------------------R----R-E-N-----------------------------------------------R----

14FSC1 ---------------------------------------------------------------------------------------------------------
14FSC2 ---F-----------------------------V-----------------------------------------------------------------------
14FSC3 ---------------------------------------------------------------------------------------------------------
14FSC7 ---------------------------------------------------------------------------------------------------------
14FSC9 ---------------------------------------------------------------------------------------------------------

14FLN1 ---H-------------------T------------N-------R-E---------------------------------------------------------
14FLN2 ---H-----------------------------V---------R-E----E-------------------------------------------------------
14FLN3 ---H-----------------------------V-N-------R-E-----------------------------------------------------------
14FLN4 ---F-----------------------------V---------R-E----E-------------------------------------------------------
14FLN5 ---------------------------------V---------R-E----E-------------------------------------------------------

13FFB2 --------I--------------M---------Q-----R----R---E---------------------------------------------------------
13FFB4 --------I--------------M---------Q-----R----R---E---------------------------------------------------------
13FFB5 --------I--------------M---------Q-----R----R---E-------------------------------R---------------------
13FFB6 --------I--------------M---------Q-----R----R---E-------------------------------R---------------------
13FFB7 --------I-----R--------M-------------------------E-------------------------------R---------------------

13FLN1 ---------------------------------------------R---E---------------------------------------------------------
13FLN2 ---------------------------------------------R---E---------------------------------------------------------
13FLN3 ------------------------------------------------E-----------I---------------------------------------------
13FLN6 ---------------------------------------------R-E---------------------------------------------------------
```

FIG. 3—*Continued*

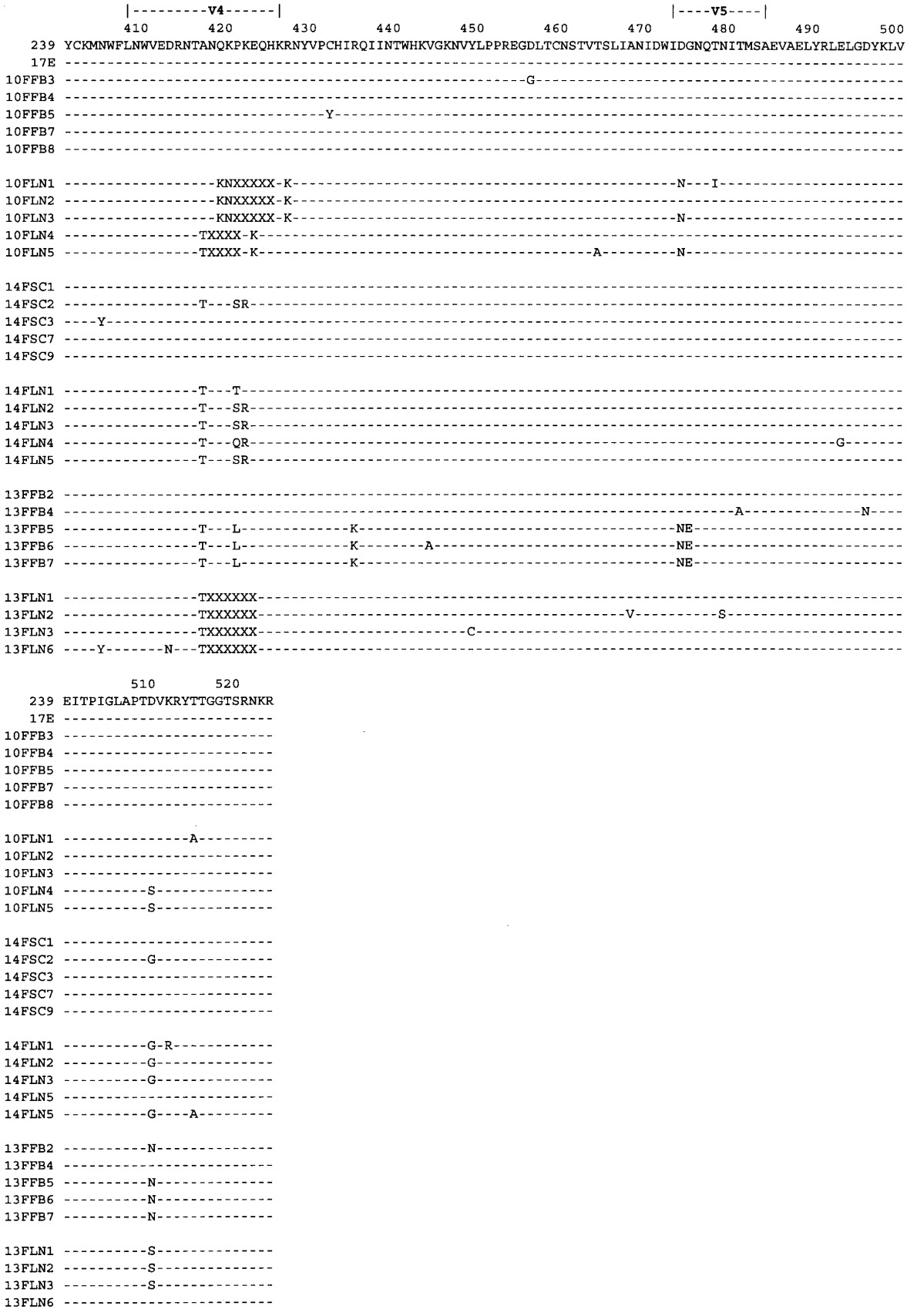


FIG. 3—*Continued*

TABLE 3

Genetic Changes in the gp120s Isolated from Macaques 10F, 13F, and 14F[a]

Animal	Tissue	No. of clones	Total No. of nucleotides sequenced	Total No. of substitutions (deletions)	Mean nucleotide substitution rate (%)	Overall mutation rate (%)	Mean change in amino acid sequence (%)
10F							
	CNS	5	7875	31(6)	0.40	0.54	1.18
	LN	5	7875	133(69)	1.68	2.56	4.48
13F							
	CNS	5	7875	115(45)	1.46	2.03	3.7
	LN	4	6300	75(78)	1.19	2.43	4.0
14F							
	CNS	5	7875	40(0)	0.51	0.51	0.99
	LN	5	7875	101(0)	1.28	1.28	3.0

[a] Viral DNA was amplified from LN and selected regions of the CNS by PCR, cloned, sequenced, and compared to $SIV_{mac}239$.

10F and 14F were from lymphocyte-tropic viruses and that the sequences from macaque 13F brain had a genotype suggestive of macrophage tropism, we constructed chimeric viruses in which the V1–V5 region of $SIV_{mac}239$ gp120 was replaced with the corresponding regions from representative clones isolated from the CNS of the three animals. The chimeric viral genomes were transfected into cultures of CEMx174 cells. Viruses constructed with V1–V5 regions from selected CNS gp120 clones from macaques 10F and 14F caused massive fusion of CEMx174 cells and produced 10^2–10^3 infectious units of cell-free virus. Chimeric viral genomes constructed with V1–V5 sequences from 13FFB2, 13FFB5, or 13FFB7 did not cause cytopathology in transfected CEMx174 cells and release of only low levels of p27 into the culture medium. Passage of the culture fluids from cells transfected with the 13F chimeric genomes did not result in an increase in the level of p27, suggesting that 13F brain constructs did not yield infectious virus. Control viruses SIV_{mac}LG1 which had macrophage-lytic properties, lymphocyte-tropic $SIV_{mac}239$ and chimeric viruses constructed with the gp120 sequences isolated from the brains of macaques 10F and 14F were used to inoculate cultures of rhesus macrophages and 5 days later the cells were examined for the presence of p27 using monoclonal antibody FA-2. As shown in Figs. 4E and 4F, macrophages infected with SIV_{mac}LG-1 had extensive staining for p27 and underwent massive syncytia formation. In contrast, macrophage cultures inoculated with $SIV_{mac}239$ had relatively few infected cells (~5% infected cells) and only rare multinucleated, infected cells (Figs. 4C and 4D). Macrophage cultures inoculated with SIV_{mac}10Fenv and SIV_{mac} 14Fenv demonstrated a pattern of p27 staining and cytopathology that was similar to $SIV_{mac}239$-infected macrophages (Figs. 4G–4J).

In addition to immunostaining for p27 antigen, inoculated macrophage cultures were radiolabeled and the viral proteins immunoprecipitated from the culture medium. Macrophage-tropic SIV_{mac}LG1 replicated very efficiently in macrophage cultures as judged by the ability to immunoprecipitate the major envelope glycoprotein (gp120) from the culture medium. In contrast, immunoprecipitation studies revealed that lymphocyte-tropic $SIV_{mac}239$ inefficiently released gp120 into the culture medium (Fig. 5). These results, used as controls here, were identical to those we had reported earlier (Stephens *et al.,* 1995). Similar to $SIV_{mac}239$, the chimeric viruses constructed with V1–V5 regions from CNS clones isolated from macaques 10F and 14F replicated very inefficiently in macrophages with only small amounts of gp120 released into the culture medium as determined by immunoprecipitation (Fig. 5). Together with the immunostaining studies, these results indicate that the chimeric viruses constructed with the V1–V5 sequences isolated from the CNS of macaques 10F and 14F were lymphocyte-tropic in nature, very similar to the parental virus $SIV_{mac}239$.

DISCUSSION

This study showed that molecularly cloned $SIV_{mac}239$, a virus that is highly lymphocyte-tropic and poorly macrophage-tropic, is highly neuroinvasive and caused persistent infection in the brain of infected macaques, without causing overt encephalitis. The infection in the CNS was probably initiated early after virus inoculation in the animals, during the phase of T-cell activation, when infected cells crossed the blood–brain barrier (Lackner *et al.,* 1991, 1994; Sharma *et al.,* 1992b). The persistent infection (as evidenced by the presence of low levels of p27 antigen in the CNS tissues taken from two of three macaques) and the slow type of replication that ensued were comparable to or reminiscent of the type of replication of this virus in macrophage cultures (Stephens *et al.,* 1995) and distinct from the events in lymphoid tissues where explosive, highly productive virus replication in

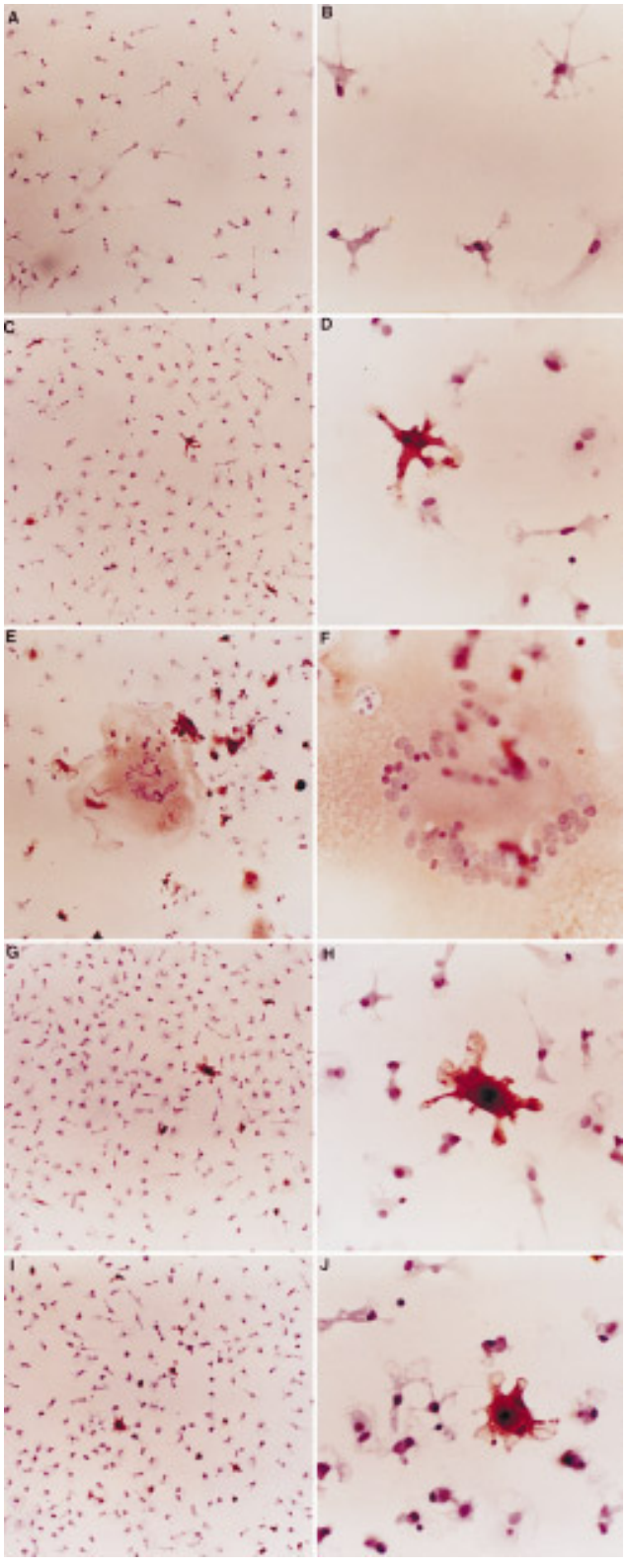


FIG. 4. Immunocytochemistry shows that the chimeric viruses constructed with V1–V5 gp120 sequences derived from the CNS of macaques 10F and 14F are lymphocyte-tropic. Macrophage cultures were infected with SIV_{mac}LG1, SIV_{mac}239, SIV_{mac}14Fenv, or SIV_{mac}23910F or were uninfected. At 5 days following inoculation, cells were washed, fixed, and stained for p27 antigen as described under Materials and Methods. The positive-staining cells have a brown color compared to the background. (A) Uninfected macrophage culture, 100× (original magnification). (B) Uninfected macrophage culture, 400× (original magnification). (C) Macrophage culture infected with SIV_{mac}239, 100× (original magnification). (D) Macrophage culture infected with SIV_{mac}239, 400× (original magnification). (E) Macrophage culture infected with SIV_{mac}LG-1, 100× (original magnification). (F) Macrophage culture infected with SIV_{mac}LG-1, 400× (original magnification). (G) Macrophage culture infected with SIV_{mac}14Fenv, 100× (original magnification). (H) Macrophage culture infected with SIV_{mac}14Fenv, 400× (original magnification). (I) Macrophage culture infected with SIV_{mac}10Fenv, 100× (original magnification). (J) Macrophage culture infected with SIV_{mac}10Fenv, 400× (original magnification).

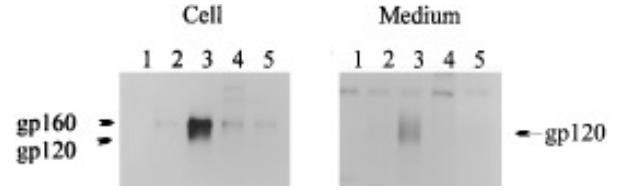


FIG. 5. Immunoprecipitation analyses demonstrate that the chimeric viruses constructed with gp120 sequences isolated from the CNS of the macaques 10F and 14F are lymphocyte-tropic. Macrophage tropism of various viruses following transfection into CEMx174 cells. Virus was harvested from transfected cultures and used to infect cultures of rhesus macrophages as described under Materials and Methods. At 5 days postinoculation, cells were starved for methionine and cysteine and then radiolabeled with 200 μCi of [^{35}S]methionine and cysteine for 18 hr. The culture medium was harvested and cell lysates were prepared. SIV proteins were immunoprecipitated using an anti-SIV antibodies and protein A–Sepharose as described in Materials and Methods. Immunoprecipitates were washed three times in RIPA buffer and samples denatured by boiling in SDS–PAGE sample-reducing buffer. Proteins were separated using SDS–PAGE (8.5% gel) and visualized using standard fluorographic techniques. The panel on the left represents SIV glycoproteins (gp120 and gp160) immunoprecipitated from cell lysates and the panel on the right represents SIV gp120 immunoprecipitated from the culture medium. Lane 1, uninfected rhesus macrophages; Lane 2, SIV proteins immunoprecipitated from rhesus macrophages infected with SIV_{mac}239; Lane 3, SIV proteins immunoprecipitated from rhesus macrophages infected with SIV_{mac}LG1; Lane 4, SIV proteins immunoprecipitated from rhesus macrophages infected with SIV_{mac}14Fenv; Lane 5, SIV proteins immunoprecipitated from rhesus macrophages infected with SIV_{mac}10Fenv.

$CD4^+$ T cells began early in the infection. The latter resulted in production of cell-free virus, development of plasma viremia, and widespread dissemination of infected, activated T cells (Joag *et al.*, 1994a). The inability to detect p27 antigen in the CNS tissues from the third macaque does not rule out the possibility that infection of macrophages was minimally productive and below the sensitivity of the antigen capture assays.

Because these animals were inoculated with a molecularly cloned virus (i.e., SIV_{mac}239) and since the *env* gene (more specifically the gp120) undergoes the most extensive variation of all viral genes in the host with time, we performed sequence analyses to determine the extent of variation the virus underwent in the brain. We then compared these with the *env* sequences isolated

from the lymph node, a tissue in which lymphocyte-tropic $SIV_{mac}239$ is known to replicate efficiently (Kestler *et al.,* 1990; Sharma *et al.,* 1992a). Since virus replication is required for accumulation of nucleotide substitutions and deletions in the viral genome over a period of time (Temin, 1993; Sanchez-Palomino *et al.,* 1993), the results presented here indicate that the virus in selected regions of the CNS from two animals had undergone considerably less replication than the corresponding virus in the lymph node. Construction and determination of the replication characteristics of chimeric viruses with the V1–V5 regions of gp120 obtained from brain of these two animals established the lymphocyte-tropic nature of virus in the brain, nearly 2 years after virus inoculation in the animal. Whether every region of CNS from macaque 10F had viral genomes with a relatively low mutation rate or whether there were specific regions of the CNS that had viral genomes with mutation rates comparable to the lymph node remains to be determined. Nevertheless, the results of this study clearly established the principle that virus with a predominantly lymphocyte tropism can cause persistent infection in the brain, an organ that has a native macrophage population, but lacks a lymphocyte population.

Our data suggest that after arrival in the brain, the virus underwent two types of replication. In macaques 10F and 14F, virus replication occurred at a slower rate in the regions sampled, with a corresponding lower mutation frequency than that of 13F and the virus maintained a lymphocyte-tropic phenotype. Genotypic analysis indicated that the viruses in these two animals were very similar to the inoculum virus. This is compatible with the idea that virus infection in the brain occurred during the early phase of infection in the animal and subsequently slowed to an extremely low level. In contrast, in macaque 13F, with a relatively low level of neural hits, subsequent virus replication in the brain evolved at a much more rapid rate. While comparison of the mutation rates of the virus in lymphoid and neural sites was similar, those developing in the brain evolved toward a more macrophage-tropic genotype. Two deductions from these observations were that, first, the brain was selecting for a macrophage viral genotype. Remarkably, the virus in the brain of macaque 13F was similar to (but not identical with) the highly neurovirulent virus that was isolated from the brain of an encephalitic macaque (17E) 4 years previously, during neuroadaptation of $SIV_{mac}239$ (Anderson *et al.,* 1993). Repeat of the selection of the selection of this particular genotype suggested specificity in the virus selection process in the brain. Second, the fact that the macrophage-tropic genotype was found only in brain and not in the lymphoid tissues of the animal suggested either that lymphocyte-tropic virus, possibly pristine $SIV_{mac}239$, entered the brain of this animal and began replicating and mutating toward a macrophage-tropic genotype or that infected macrophage precursors from other tissues such as the lung or the bone marrow could have deposited this macrophage-tropic viral genome in the brain.

The identification of *env* genes of virus with lymphocyte-tropic and macrophage-tropic genotypes in the brain provided an opportunity to examine markers of macrophage tropism. Previous studies claimed that selection of macrophage-tropic variants of SIV_{mac} was associated with certain amino acid substitutions (Mori *et al.,* 1992). One of these substitutions, a valine to methionine change at position number 67, has been implicated as a genetic marker for the for selection of macrophage-tropic variants of SIV_{mac} (Mori *et al.,* 1992). Our studies here suggest clearly that this mutation merely represented a marker for virus replication in macaque tissues, irrespective of whether the virus is a lymphocyte-tropic, 239-like virus or a virus that is capable of productive replication in macrophages. This is supported by the sequence analysis of gp120 clones isolated from the CNS of macaques 14F and 10F. Those gp120 clones from the CNS that had a low mutation rate (and presumably did not undergo as many rounds of replication) did not have the valine to methionine/leucine substitution. In contrast, the 13F brain and lymph node viruses that had a high rate of mutation had this valine to methionine/leucine substitution. The lack of a role for the valine to methionine change at position 67 is further indicated by the analysis of chimeric viruses. The chimeric virus, $SIV_{mac}LG1$, used in this study is both highly macrophage-tropic and cytopathic for rhesus macrophages in culture yet the valine at position 67 was retained in this virus. Further, in a recent study of the chimeric virus $SIV_{mac}239$/17E, we used chimeric viruses to demonstrate that the valine to methionine substitution was not important for the macrophage tropism of this virus (Stephens, unpublished observations). In that study, a glutamine to asparagine change at position 217 and a methionine to isoleucine change at position 309 were found to determine macrophage-tropic properties of $SIV_{mac}239$/17E.

In summary, these studies have shown that following inoculation into macaques, $SIV_{mac}239$ will invariably undergo productive replication in lymphoid tissues, but accompanying mutations maintain lymphocyte-tropic genotypes. The virus is highly neuroinvasive, but its rate of replication in the CNS may be either similar to that in lymphoid tissues or it may replicate at an extremely slow rate. In the former case, illustrated by macaque 13F, despite the similarity in rates of mutation, the viruses that evolved had distinct genotypes, the lymph node virus being lymphocyte-tropic and the brain virus evolving toward a macrophage-tropic genotype. The surprising data came from examination of the slowly replicating virus in the brains of 10F and 14F. Here, the rates of virus replication were not only divergent between brain and lymphoid

tissue, but the brain virus maintained the pristine genotype of the inoculum virus, whereas the lymph node virus mutated away, although maintaining its lymphocyte tropism. This clearly illustrated that highly lymphocyte-tropic virus could invade the CNS and cause a persistent infection lasting years, without mutating to a macrophage-tropic genotype.

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