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Neurovirulent Simian Immunodeficiency Virus Replicates Productively in Endothelial Cells of the Central Nervous System In Vivo and In Vitro

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The perivascular location of human immunodeficiency virus-infected cells suggests that the virus enters the central nervous system (CNS) by traversing the blood-brain barrier (BBB). In this study, the simian immunodeficiency virus (SIV) macaque model was used to determine whether SIV infects CNS endothelial cells. SIV RNA was detected in capillary endothelial cells in brain sections from animals parenterally inoculated with a neurovirulent strain of SIV by double immunohistochemistry and in situ hybridization and by reverse transcriptase-in situ PCR. These in vivo observations were extended by examining whether SIV replicated productively in cultured macaque brain endothelial cells (MBEC). A neurovirulent strain, SIVmac239/17E-Br, replicated productively in MBEC as determined by the presence of viral cytopathic effect (syncytia), viral DNA by PCR, viral RNA by in situ hybridization, and viral antigen by immunohistochemistry and by the production of high titers of cell-free virus. Virus replication was confirmed by electron microscopy. In contrast, a nonneurovirulent strain, SIVmac239, did not infect MBEC. Infection of the endothelial cells was not blocked by soluble CD4. Thus, endothelial cells may provide a CD4-independent pathway of virus entry to the CNS. In addition, damage to the BBB as a result of endothelial cell infection may provide a mechanism for amplification of viral load in the CNS and may contribute to the CNS dysfunction that characterizes AIDS dementia and SIV encephalitis. These data suggest that MBEC may serve a selective role in determining virus entry to the CNS.

Despite the high prevalence of neurological disease in human immunodeficiency virus (HIV)-infected people, the mechanism of viral entry into the central nervous system (CNS) and the pathogenesis of CNS disease are not well understood. The perivascular location of HIV-infected cells strongly suggests that the virus enters the CNS by traversing the blood-brain barrier (BBB) either as cell-free virus or in virus-infected cells (2, 3, 20, 26). The BBB consists of endothelial specializations (i.e., tight junctions and absent vesicular transport) that limit the transendothelial and interendothelial movement of water-soluble compounds and cells from blood to brain (4, 7, 12). Several studies have demonstrated the presence of serum proteins in the brain parenchyma of patients with AIDS dementia complex, providing evidence of alterations in the integrity of the BBB in HIV infection (14, 15, 17). The perivascular location of the virus and the evidence of BBB alterations in HIV encephalitis suggest that endothelial cells, as the principal component of the BBB, may play a role in the pathogenesis of HIV CNS disease. Several reports have demonstrated HIV RNA or proteins in brain endothelial cells in autopsy material from patients with AIDS dementia (18, 27), but this finding has not been universal (8, 23). Virus replication in endothelial cells would not only provide a mechanism for initial viral entry to the CNS, but virus-induced changes in endothelial cells could potentially alter the integrity and function of the BBB. Virus-induced alteration of the expression of cellular adhesion molecules located on the endothelial cells, for example, may promote the influx of inflammatory cells, some of which would be infected. Such BBB alterations could, in turn, result in an increased virus load in the CNS and disturb the homeostatic balance within the neuropil, thereby contributing to the CNS dysfunction reported in HIV-infected individuals.

Simian immunodeficiency virus (SIV) infection of rhesus macaques is an excellent model to study the pathogenesis of HIV CNS disease. Rhesus macaques infected with SIVmac develop motor and cognitive deficits similar to those identified in HIV-1-infected patients (11), and the pathological changes in the CNS of macaques are similar to those in HIV encephalitis, with perivascular, virus-laden macrophages and multinucleated giant cells (19). The SIV macaque model has facilitated the study of the molecular basis of lentivirus disease because it is possible in animals to examine the potential of defined virus strains to cause disease. The purpose of this study was to determine whether endothelial cells, a vital component of the BBB, are infected in animals with SIV-induced neurological disease. Replication of neurovirulent strains of SIV in endothelial cells was demonstrated both in vivo and in vitro.

MATERIALS AND METHODS

Macaques. Yearling rhesus macaques were obtained from the breeding colony at this institution. Animals were prescreened for antibodies to SIV and to type D retroviruses. Macaques were inoculated with SIV into the femoral bone marrow while under general anesthesia with halothane. Bone marrow inoculation is used to provide ready access of virus to

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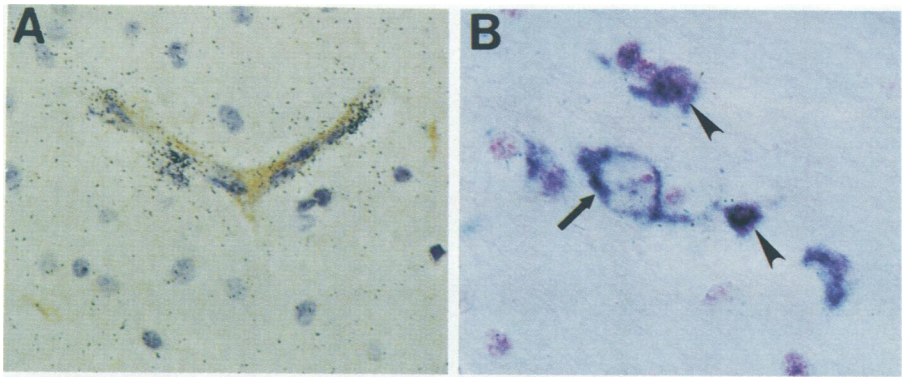


FIG. 1. Brain tissue from a macaque with SIV encephalitis 6 months after inoculation with a neurovirulent strain of SIV. Tissue double labeled with antibody against factor VIII-related antigen and by in situ hybridization to detect viral RNA demonstrated SIV RNA in endothelial cells (A). Viral RNA was demonstrated by reverse transcriptase-in situ PCR (B) both in perivascular macrophages (arrowheads) and in cells encircling the lumen of a blood vessel (arrow). Magnification, ×400.

macrophage precursors, although most of the virus enters the vascular system by this route. After development of neurological disease, animals were euthanized by exsanguination while under deep anesthesia. Brain tissue was fixed in 10% buffered formalin and embedded in paraffin.

Virus. Previous studies showed that multiple sequential intracerebral passage of a cloned, lymphocyte-tropic, nonneurovirulent strain of SIV, SIVmac239, in the brains of rhesus macaques resulted in the selection and replication of a macrophage-tropic, neurovirulent strain, SIVmac239/17E-Br (21) that differed from the parental virus by 16 amino acids (1). SIVmac239/17E-Br, a macrophage-tropic, neurovirulent strain of SIV derived from SIVmac239 by multiple intracerebral passage in macaques, or SIVmac239, a cloned lymphocyte-tropic, nonneurovirulent strain (1, 21), was used for in vitro studies. SIVmac239/R71, a closely related neurovirulent virus with the same *env* consensus sequence as SIVmac239/17E-Br, was used in the animal studies, which were performed retrospectively. Virus stocks were expanded by inoculation of CEMx174 cells. The supernatant fluid had titers of 10^3 50% tissue culture infectious doses when assayed in CEMx174 cells.

MBEC. Approximately 20 g of macaque cortical gray matter was minced and passed through 20-gauge needles to dissociate the neuropil, followed by passage through 320- and 118-μm nylon mesh filters. Myelin was removed from the homogenate by centrifugation on 20% dextran, and the pelleted material was then passed over a 53-μm nylon mesh. The trapped microvessels were agitated with 2% collagenase-dispase (Boehringer-Mannheim) at 37°C for 20 min to digest the basal lamina and release pericytes, and the remaining microvessels were cultured on plastic flasks coated with human fibronectin in minimum essential medium containing D-valine substituted for L-valine (92 mg/liter) with 15% human platelet-poor plasma–5% fetal bovine serum–endothelial growth factor (0.05 mg/ml; Biomedical Technologies Inc.) at 37°C and 5% CO_2. Macaque brain endothelial cell (MBEC) cultures consisted of greater than 99% endothelial cells as determined by immunohistochemical staining with factor VIII-related antigen. The presence of macrophages or astrocytes in the cultures was excluded by immunocytochemical staining for CD68 (EBM-11; DAKO) and GFAP (DAKO), respectively. Stringent washing (six times) served to remove nonadherent cells, including lymphocytes. Cultures did not include any cells of the size and rounded morphology of lymphocytes. The cells were passed four times and frozen in aliquots for use in this study.

Inoculation of MBEC. MBEC were inoculated with SIVmac239/17E-Br at a multiplicity of infection of 0.01, the virus inoculum was removed after 24 h, the cells were washed vigorously with minimum essential medium containing D-valine substituted for L-valine (92 mg/liter) six times, and the medium was replaced. Thereafter, the supernatant was removed daily for measurement of cell-free virus, the cultures were again washed six times, and the medium was replaced. At 22 days postinoculation (p.i.), the cells from some cultures were trypsinized and cytocentrifuge preparations were made for the detection of viral RNA by in situ hybridization and of viral antigen by immunocytochemistry. Cells from additional flasks were lysed and prepared for DNA amplification by PCR, and other cells were fixed in 3% glutaraldehyde in cacodylate buffer for electron microscopy.

Immunohistochemistry and lectin histochemistry. The following reagents were used: RCA-1 (Vector) to identify macrophages, anti-FVIII-related antigen (Endotech) to identify endothelial cells, EMB-11 (DAKO) to identify macrophages, kk41 (gift of K. Kent) to detect the SIV transmembrane protein, and anti-GFAP (DAKO) to identify astrocytes. Immunohistochemistry was performed by using the avidin-biotin complex technique with peroxidase as the substrate for color reaction with 3,3′-diaminobenzidine tetrahydrochloride. Appropriate isotype controls were used. Lectin histochemistry was performed with biotinylated lectin on tissues pretreated with trypsin (0.025% in phosphate-buffered saline) at room temperature for 5 min. Double-labeled slides were stained immunohistochemically first, followed by in situ hybridization within a week.

In situ hybridization. Brain sections or cytospin preparations were pretreated with 0.2 N HCl–25 μg of proteinase K per ml, acetylated, dehydrated, and air-dried as previously described (28). In situ hybridization was performed by using a full-length molecular clone of SIVmac239 provided by R. Desrosiers that was radiolabeled by random priming with [^{35}S]dCTP (Amersham Corp.). Radiolabeled DNA (0.2 μg/ml) was denatured, and hybridization was performed for 16 h at 25°C. The slides were washed, dehydrated, dipped in NTB3 autoradiographic emulsion (Eastman Kodak Co.), air-dried, and developed after 3 to 10 days of exposure in the dark. Uninfected and SIV-infected macaque tissues and CEMx174 cells and a nonspecific radiolabeled probe were used as controls.

Reverse transcriptase-in situ PCR. Tissues were permeabi-

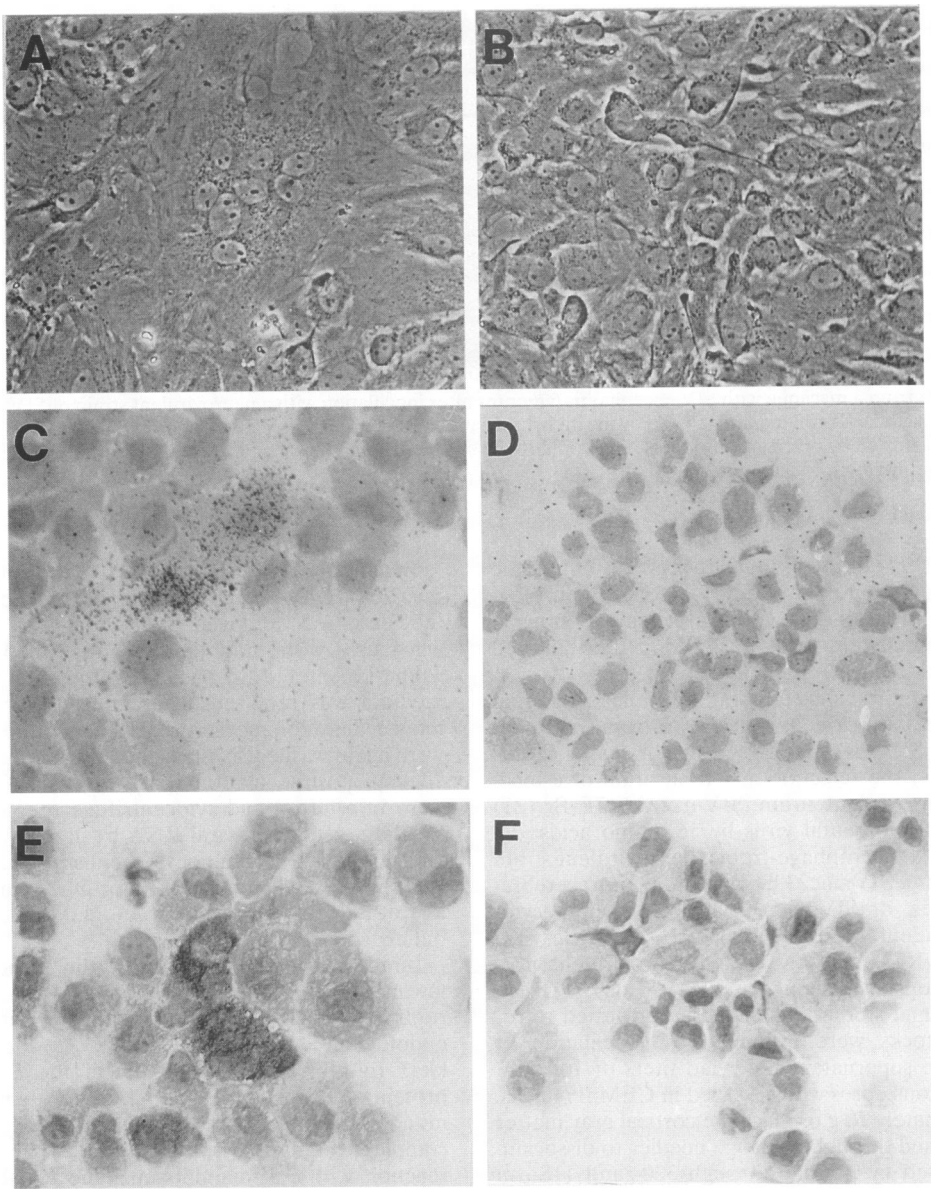


FIG. 2. MBEC cultures inoculated with SIVmac239/17E-Br (A, C, and E) or SIVmac239 (B, D, and F). Cultures inoculated with SIVmac239/17E-Br developed syncytia 7 days after inoculation (A) and contained viral RNA, as demonstrated by in situ hybridization (C), and viral protein, as detected by immunocytochemistry (E). MBEC cultures inoculated with SIVmac239 did not develop syncytia (B) and did not contain viral RNA (D) or protein (F). Magnification, ×200 (A and B) and ×400 (C to F).

lized with 10 μg of proteinase K (Boehringer-Mannheim) per ml at 37°C for 15 min. Extant DNA was removed by incubation in 750 U of DNase I per ml overnight at 22°C. cDNA was synthesized by utilizing 19.2 U of avian myeloblastosis virus reverse transcriptase–0.4 μg of oligo(dT) at 42°C for 1 h. Hot-start PCR was performed under a sealed glass coverslip by using 2.5 mM $MgCl_2$–10× PCR buffer (Boehringer-Mannheim)–5 U of *Taq* polymerase (Boehringer Mannheim)–2 μM primers 5′-AAGCTTGGATCCCTCCAACGAGCGCTCCTTCAT, 3′-CACTTAAAAGCAAGATCGCGATAAGCA that amplified a 309-bp portion at the 3′ end of the *env* gene of SIV. Three cycles of 90°C for 1 min, 55°C for 30 s, and 72°C for 30 s were followed by 27 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s. After amplification, hybridization was performed overnight at 55°C with a digoxigenin-labeled 21-bp oligonucleotide probe (AACAAAACTGGCAATGGTAGC) which was complementary to sequences internal to the two primers. Hybridized probe was detected by using Nitro Blue Tetrazolium as the substrate, and sections were counter-stained with nuclear fast red.

PCR. DNA was isolated from MBEC by phenol extraction and ethanol precipitation. Primers (5′-AAGCTTGGATCCCTCCAACGAGCGCTCCTTCAT; 3′-CACTTAAAAGCAAGATCGCGATAAGCA), complementary to bases 6343 to 6375 and 6652 to 6626, were used with 35 cycles to amplify a 309-bp segment from the 3′ end of the *env* gene. Ampliwax (Perkin-Elmer Cetus) was used to separate the DNA template from the reaction components before cycling. The reaction product was detected by Southern blot.

Viral titration and antigen capture. Supernatants obtained

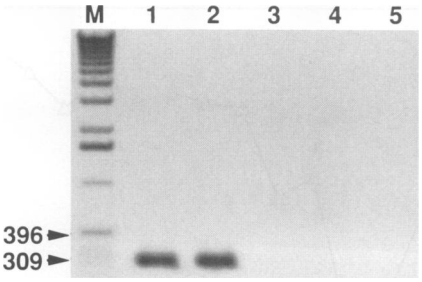


FIG. 3. Southern blot of PCR-amplified DNA extracted from MBEC inoculated with SIVmac239/17E-Br or SIVmac239. CEMx174 cells inoculated with SIVmac239 (lane 1), MBEC inoculated with SIVmac239/17E-Br (lane 2), MBEC inoculated with SIVmac239 (lane 3), mock-inoculated MBEC (lane 4), and no DNA (lane 5).

directly from MBEC cultures were harvested daily for the first 15 days p.i. and evaluated for infectious virus by coculturing half-log dilutions with indicator CEMx174 cells and monitoring for cytopathic effect. Cell-free supernatant concentrations of the core antigen p27 were determined by utilizing an enzyme immunoassay employing a murine monoclonal antibody directed against the core antigen p27 (Coulter).

CD4 blocking. Inoculum virus was pretreated for 1 h with 20 μg of soluble CD4 (sCD4) per ml, a concentration that abrogated infection of highly CD4-positive CEMx174 cells by SIVmac239/17E-Br. MBEC were then inoculated with the pretreated SIVmac239/17E-Br and maintained in medium containing 20 μg of sCD4 per ml. The cultures were observed daily for the development of cytopathic effect (syncytia), and virus in cell free-supernatants was titrated by cocultivation on CEMx174 cells.

RESULTS

Detection of SIV in endothelial cells in vivo. Brain sections were examined from animals infected with a neurovirulent strain of SIV to determine which cells supported productive viral replication. Macrophages containing viral RNA were seen in perivascular cuffs and in the surrounding neuropil by in situ hybridization. Double-labeling studies demonstrated viral RNA in cells that stained immunohistochemically for factor VIII-related antigen, a specific endothelial cell marker (Fig. 1A).

To confirm the presence of viral gene products in endothelial cells in vivo, reverse transcriptase-in situ PCR was employed as a more sensitive technique for the detection of viral RNA than routine in situ hybridization. Digoxigenin-labeled probes also provided improved subcellular localization of the amplified target. Brain sections from a macaque which had been inoculated with a neurovirulent strain of SIV and which had typical encephalitis contained viral RNA in two distinct perivascular locations in the brain: in large cells resembling perivascular macrophages and in a thin rim around the lumen of the blood vessels, an area that corresponded morphologically with the cytoplasm of endothelial cells (Fig. 1B). These changes were not seen in animals inoculated with a nonneurovirulent strain of SIV, SIVmac239. These in vivo studies confirmed that viral RNA is present in perivascular macrophage-like cells and suggested that neurovirulent strains of SIV also replicate in endothelial cells.

Infection of endothelial cells in vitro. The in vivo observations were extended by examining if SIV replicated productively in primary macaque brain endothelial cell cultures. The neurovirulent strain SIVmac239/17E-Br replicated productively in cultured CNS-derived microvascular endothelial cells (MBEC), whereas the nonneurovirulent SIVmac239 did not. Syncytia appeared in the cultures of MBEC inoculated with SIVmac239/17E-Br (Fig. 2A) at 7 days p.i. and were present throughout the observation period (22 days). MBEC cocultivated with CEMx174 indicator cells developed cytopathic effect. MBEC inoculated with SIVmac 239/17E-Br contained SIV RNA by in situ hybridization (28) (Fig. 2C) and were also positive for viral antigen by immunocytochemical staining using a monoclonal antibody directed against the viral transmembrane protein (Fig. 2E). In addition, viral DNA was shown to be present in MBEC inoculated with SIVmac239/17E-Br by amplification of a 309-bp segment of DNA at the 3′ end of the *env* gene by PCR (Fig. 3). Cultures inoculated with SIVmac239 did not develop syncytia (Fig. 2B), and these cells did not produce cytopathic effect when cocultivated with indicator cells. SIVmac239-inoculated cells were negative for RNA (Fig. 2D) and for viral antigen (Fig. 2F) and did not contain viral DNA (Fig. 3).

In these cultures, which were washed stringently each day to remove free virus and degenerating virus-infected cells, approximately 1 in 100 cells inoculated with SIVmac239/17E-Br was positive by in situ hybridization and immunohistochemistry. This number was later confirmed by electron microscopic examination. Other MBEC cultures were inoculated with identical viral stocks of SIVmac239/17E-Br at equivalent multiplicities of infection and were not washed, thus permitting accumulation of the virus in the cultures. In these cultures, all cells had formed syncytia and lysed by 17 days p.i. Thus, all MBEC were susceptible to infection with the neurovirulent strain.

A viral growth curve was obtained from infected MBEC over a 2-week period by titration of cell-free culture supernatants on CEMx174 cells (Fig. 4A). The end point was determined with the Spearman-Kaeber formula. During the first 2 days of culture, small amounts of residual inoculum virus remained in the supernatant. Newly elaborated virus was detected on day 4 p.i. in the supernatant of cultures inoculated with SIVmac239/17E-Br, and viral replication continued over the next 11 days, peaking at 13 days p.i. No virus was produced by MBEC inoculated with SIVmac239. A similar pattern of replication was seen when virus production was measured by antigen capture (Coulter) for SIV p27 (Fig. 4B).

Transmission electron microscopy confirmed replication of SIVmac239/17E-Br in MBEC. Viral particles were readily identified in cytoplasmic invaginations or vacuoles in MBEC inoculated with SIVmac 239/17E-Br (Fig. 5A). The ultrastructural appearance of these virions, with dense, oblong cores and prominent glycoprotein surface projections, was consistent with the morphologic characteristics of SIV infection. Virions budding into these vacuoles were also seen commonly (Fig. 5B). No viral particles were detected in MBEC inoculated with SIVmac239. Together, these studies documented that SIVmac239/17E-Br was capable of entering MBEC, undergoing reverse transcription to proviral DNA, being transcribed to RNA, and being translated to yield viral protein and free virions.

Effect of sCD4 on virus entry to MBEC. SIVmac239/17E-Br treated with sCD4 at 20 μg/ml preinoculation remained fully capable of infecting MBEC. Infection of highly $CD4^+$ CEMx174 cells by SIVmac239/17E-Br was abrogated by pretreatment of virus with sCD4 at the same concentration. MBEC cultures inoculated with SIVmac239/17E-Br which had been treated with 20 μg of sCD4 per ml preinoculation developed syncytia, and supernatant fluids from sCD4-treated

and -untreated cultures both contained high titers of virus (10^3 and 10^4, respectively). These studies demonstrated that infection of MBEC by SIVmac239/17E-Br was not CD4 dependent.

DISCUSSION

These data have shown both in vivo and in vitro that the CNS endothelial cell is a target cell for replication of neurovirulent strains of SIV. Other retroviruses have been shown to be capable of infecting CNS endothelial cells, including neurovirulent strains of both Moloney and Friend murine leukemia viruses (MuLVs). Replication of the neurovirulent PVC-211 strain of MuLV in the CNS is restricted to brain endothelial cells, and this leads to astrogliosis, neuropil vacuolation, and widespread neuronal degeneration (5). The endothelial cell is also the major host cell for replication of the neurovirulent TR1.3 strain of Friend MuLV that causes stroke-like lesions in mice (13). The neurovirulent *ts*1 strain of Moloney MuLV has a broader cell tropism in the CNS, but a correlation has been shown between high levels of virus replication in capillary endothelial cells and the characteristic spongiform degeneration that is seen in infected animals (25). In these viruses and in SIV, the CNS endothelial cell may serve as the initial determinant for selection of neurovirulent viruses. These cells may select for viruses that can invade the CNS and then subsequently infect other neural cells such as microglia. Thus, a tropism for endothelial cells in vitro might predict neurovirulence in vivo. In this study, the neurovirulent SIVmac239/17E-Br strain exhibits an in vitro tropism for MBEC, unlike the nonneurovirulent SIVmac239, supporting the validity of this paradigm.

The brain microvascular endothelial cells play a critical role in the maintenance of the BBB. This barrier regulates the influx of molecules into the brain and protects CNS tissue from exposure to many foreign organisms. Recent studies demonstrating the presence of serum proteins in the CNS parenchyma of patients with AIDS dementia complex have provided good evidence of alterations in the integrity of the BBB during infection with HIV-1 (14, 15, 17). There have also been reports of BBB alterations during infection of macaques with SIV (22). Infection of brain microvessel endothelial cells may contribute to the development of CNS disease both directly and indirectly. Infection of endothelial cells may result in spread of virus to surrounding cells, including perivascular macrophages, thus providing a direct mechanism for virus entry to the brain. In addition, infection may alter the expression of cell adhesion molecules on endothelial cells or alter the cells' barrier properties, leading to an influx of inflammatory cells and amplification of the viral load in the CNS. Further, virus-induced alteration of BBB integrity may contribute to the CNS dysfunction that characterizes AIDS dementia and SIV encephalitis.

There are several reports of detection of HIV gene products in endothelial cells in CNS tissues from patients with AIDS (18, 27), although the finding of HIV in endothelial cells has not been universal (8, 23). A recent report demonstrated productive infection of cultured human brain microvessel endothelial cells by HIV-1 (10). Similar to our findings, these investigators demonstrated that viral infection of endothelial cells was not mediated by the CD4 molecule. This is consistent with the reported lack of CD4 expression on CNS endothelial cells (10). However, in contrast to our data that show a correlation between macrophage tropism, endothelial cell tropism, and neurovirulence, those investigators demonstrated productive infection of human brain endothelial cells only with lymphocyte-tropic strains of HIV-1 and not with macrophage-tropic variants (11a). This apparent disparity may be due to differences in the replication efficiency of the macrophage and lymphocyte-tropic HIV strains or differences in the culture conditions for these viruses in vitro.

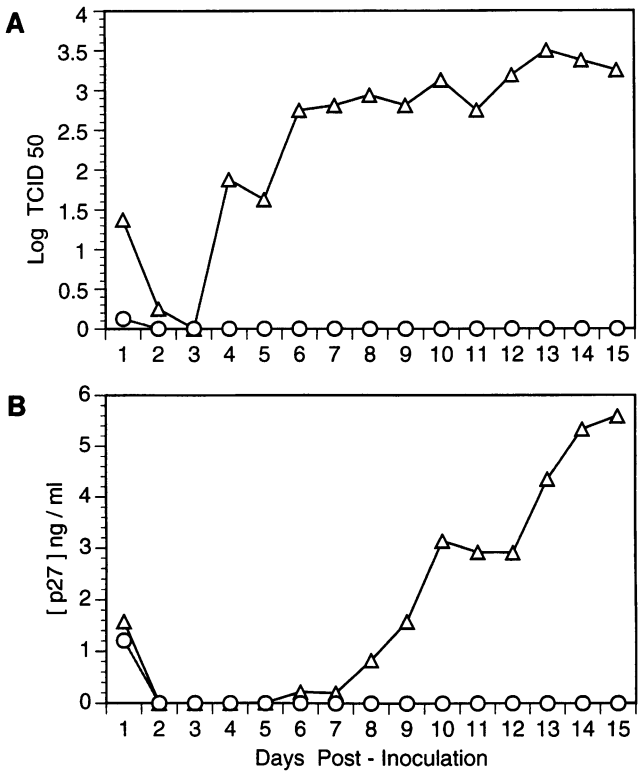
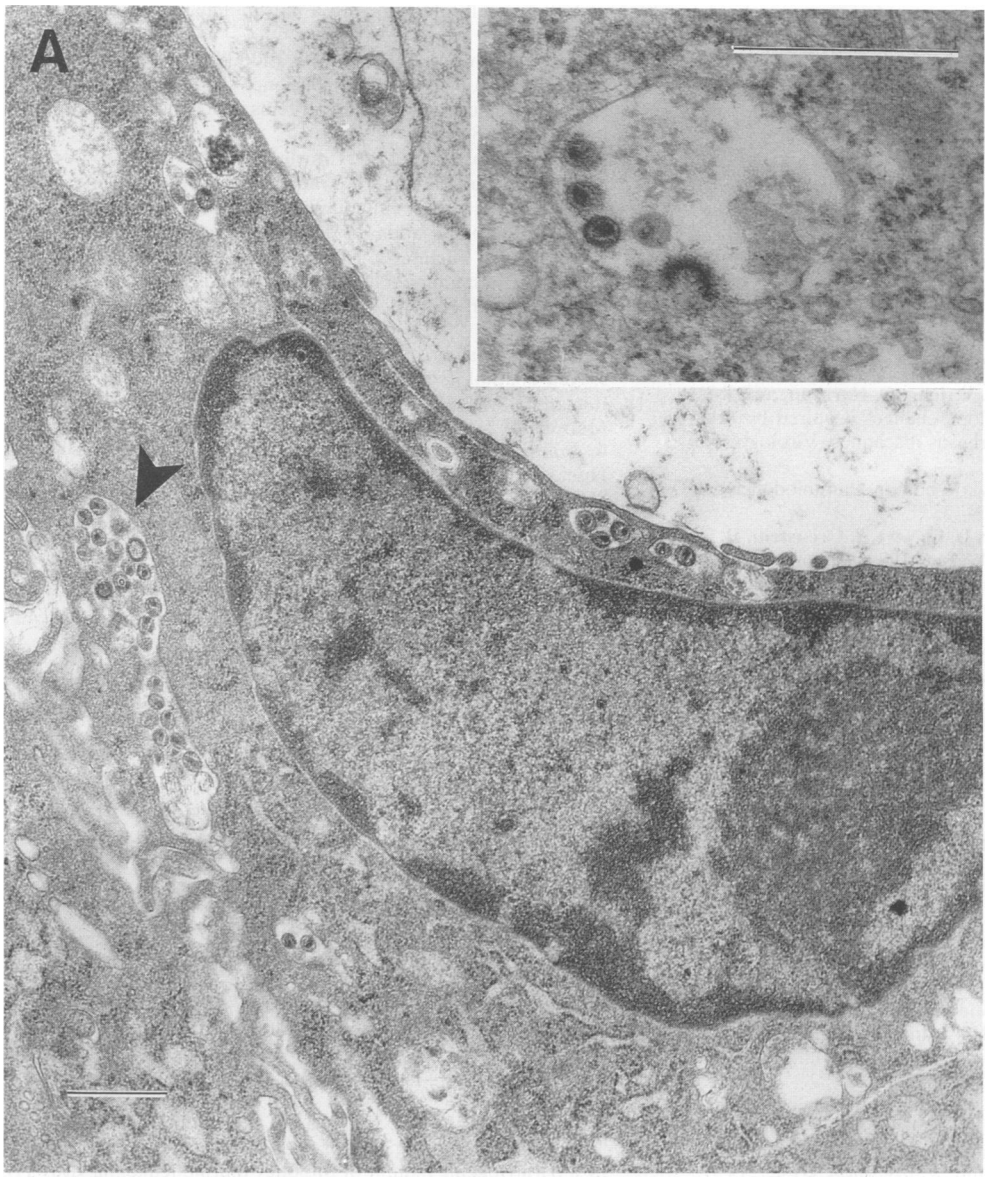


FIG. 4. Kinetics of virus production in MBEC inoculated with SIVmac239/17E-Br or SIVmac239. (A) MBEC inoculated with SIVmac239/17E-Br (△) showed a steady increase in the production of cell-free virus from day 3 p.i. in contrast to MBEC inoculated with SIVmac239 (○) which contained no infectious virus in supernatants after removal of residual inoculum virus after day 2 p.i. (B) A similar pattern was revealed by measuring viral antigen (p24) in culture supernatants.

Virus can be clearly seen budding internally into vacuoles of MBEC inoculated with SIVmac239/17E-Br by electron microscopy. This internal budding pattern is also seen in macrophages infected with HIV and SIV. That the endothelial cell-tropic strain of SIV used in these studies was also macrophage-tropic suggests that this virus may use a similar pathway for final viral processing and assembly.

Studies have demonstrated various abilities of endothelial cells derived from different tissues to support productive infection with HIV-1. Cultured endothelial cells from hepatic sinusoids and adipose tissue previously have been shown to support replication of HIV in contrast to endothelial cells derived from human umbilical or saphenous vein preparations (9, 16, 24). The susceptibility to HIV infection exhibited by different endothelial cell types does not necessarily correlate with expression of the CD4 receptor (10, 16). In addition, transfecting CD4 into umbilical endothelial cells does not confer susceptibility to infection (6). The proven phenotypic differences between endothelial cells derived from the CNS microvasculature and from nonneural vasculature highlight the importance of utilizing primary organ-specific endothelial cell cultures to establish this correlation between in vivo and in vitro findings. Further characterization of the receptor which



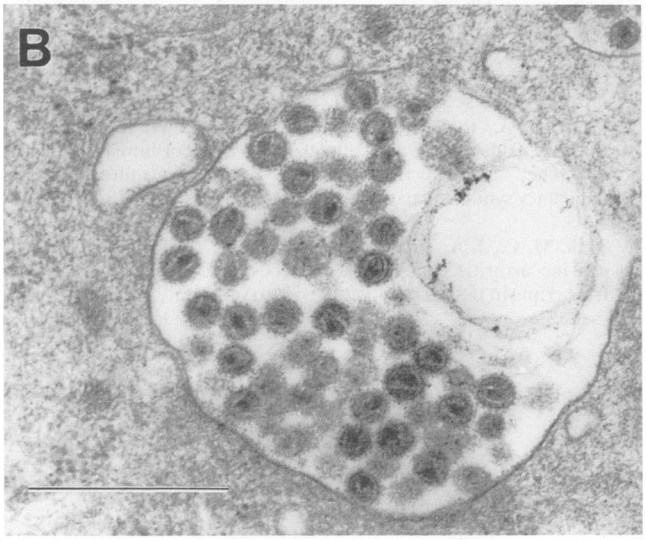


FIG. 5. Electron microscopy of MBEC inoculated with SIVmac239/17E-Br. (A) Viral particles are present in cytoplasmic invaginations or vacuoles (arrowhead) of MBEC inoculated with SIVmac 239/17E-Br. (B) The ultrastructural appearance of virions, with dense, oblong cores and prominent glycoprotein surface projections, is consistent with the morphologic characteristics of SIV. (Inset) Virions budding into these vacuoles were also seen commonly. Bars, 0.5 μm.

SIVmac239/17E-Br employs to gain entry to macaque brain endothelial cells will be vital for assessing the role that lentivirus infection of CNS endothelial cells plays in modulating entry of virus into the CNS and in altering BBB integrity.

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