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Derivation of Neurotropic Simian Immunodeficiency Virus from Exclusively Lymphocytetropic Parental Virus: Pathogenesis of Infection in Macaques

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Neurological disease resulting from lentivirus (including human immunodeficiency virus) infections is usually caused by a strain of virus that replicates productively in microglia in vivo and in macrophage cultures in vitro. We undertook this study using the model of simian immunodeficiency virus in macaques (SIVmac) to test the hypothesis that macrophage tropism is a prerequisite for neurotropism of the virus. Using molecularly cloned SIVmac239, a virus which is lymphocyte- but not macrophagetropic, we showed that this virus failed to infect brain after intracerebral (i.c.) inoculation into two macaques. Rather, these inoculations resulted in disseminated infection in lymphoid organs and the bone marrow. Two sequential passages of infected bone marrow cells inoculated i.c. into new macaques resulted in severe neurological disease and classical neuropathological lesions. Virus obtained from affected brain answered the hypothetical question: it was neurotropic and macrophagetropic. New findings in the study were that both lymphocyte- and macrophagetropic viruses were present in the animals, but the viruses localized in different tissues: the lymphotropic virus in the spleen, lymph nodes, and plasma and the macrophagetropic virus in the brain and lungs. To determine whether the brain virus was preferentially neurotropic and whether it had neuroinvasive properties, infectious brain homogenate was inoculated into one animal i.c. and into two others peripherally. The i.c. inoculated animal developed fatal encephalitis 5 months later, and examination of tissues showed cell-free virus only in brain homogenates. Only microglia were infected despite persistent viremia and infection in bone marrow cells. The two macaques inoculated peripherally remained healthy and were euthanized at 6 months. Virus replication was detected only in the bone marrow cells and peripheral blood mononuclear cells. No infection in any macrophage population in visceral organs was detected, and the virus did not invade the brain. The strictly microglial specificity of this virus suggested that different macrophage populations in the body may select specific phenotypes of lentivirus from the quasispecies of virus in the bone marrow. This could provide the basis for specific disease affecting different organ systems.

Simian immunodeficiency virus of macaques (SIVmac) causes pathological changes and clinical diseases in macaques that are strikingly similar to the results of human immunodeficiency virus (HIV) infection in human beings (8, 19, 29). The macaque model thus provides a useful system for exploring the mechanisms of pathogenesis of various clinical syndromes associated with HIV infection. Both SIV and HIV are members of the lentivirus family of retroviruses (28); they cause persistent infection and infect mainly helper T lymphocytes and cells of the macrophage lineage in vivo (5, 7, 8, 11, 20). The T cells gradually become depleted during persistent infection, and this leads to profound immunosuppression, which potentiates infections with multiple opportunistic agents (11). In addition, many hosts develop lentivirus-induced pneumonia and encephalopathy, which are associated with massive virus replication in resident macrophages in the lungs and brain (14, 16, 24, 28, 30). This suggests that viruses with a strict tropism for lymphocytes may cause mainly AIDS, whereas viruses with a tropism for macrophages may cause organ-specific diseases such as pneumonia and encephalopathy (5, 17, 25). SIVmac infection in macaques provided an ideal model system for testing this hypothesis. Not all molecular clones of this virus are pathogenic (21). Cloned, pathogenic SIVmac239 was chosen for this experiment because the virus replicates only in lymphocytes and not macrophages. We and others (9, 13, 31) showed that this agent caused disease associated only with depletion of T cells. We have extended these studies by investigating the pathogenesis and phenotypic markers of a neurotropic variant derived from SIVmac239. Unique conditions for derivation of this variant virus are described. Whereas the exclusively lymphocyte-tropic SIVmac239 failed to replicate in brain, the variant virus that had neurotropic properties was highly macrophagetropic.

We have used the terms "neurotropic" to mean virus that is capable of replicating in brain, "neurovirulent" to mean virus that causes neurological disease, and "neuroinvasive" to mean virus that spreads naturally from nonneural sites to the brain (12).

MATERIALS AND METHODS

Preparation of SIVmac239. Macaque phytohemagglutinin-induced blasts (peripheral blood mononuclear cells cultivated in lymphocyte activation medium [LAM]; see below) were transfected with molecularly cloned DNA of SIV mac239, kindly provided by R. Desrosiers, Harvard University (31). The supernatant fluid had titers of 10^4 and 10^5 50% tissue culture infective doses ($TCID_{50}$) when assayed in macaque phytohemagglutinin-induced blasts and C8166

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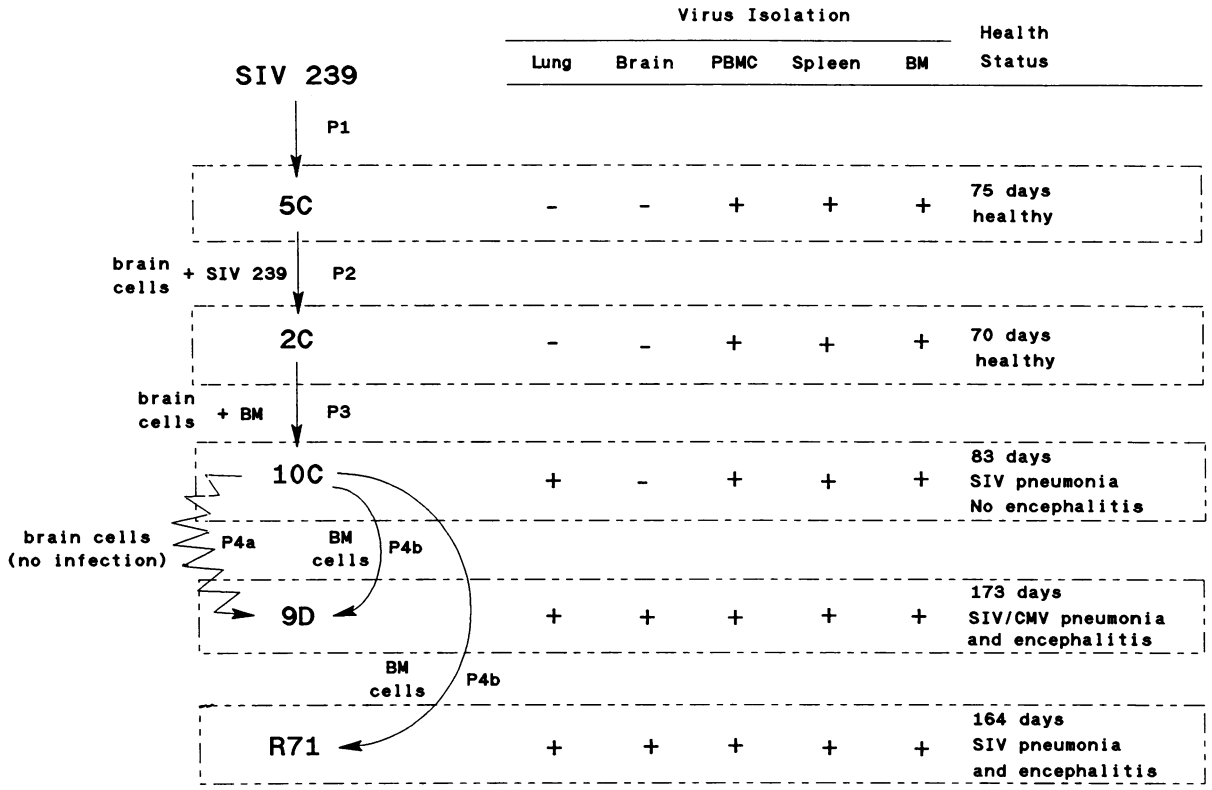

FIG. 1. Tissue dissemination of SIV during sequential i.c. passage of virus 239 and tissue cells in macaques. +, positive infection, −, no infection; P, passage. Macaques 5C and 2C were inoculated with virus 239, 10C was inoculated with BMC of 2C (BM), and 9D was inoculated first with brain cells from 10C. Later, 9D and R71 were inoculated with BMC of 10C. All inoculations were given i.c. CMV, cytomegalovirus.

cells, respectively, and 10^1 $TCID_{50}$ in primary macaque macrophages.

Neuroadaptation of SIVmac239. The procedure used to obtain a neurotropic variant (neuroadaptation) of the lymphocytetropic SIVmac239 initially followed classical virological procedures (Fig. 1). The virus was inoculated intracerebrally (i.c.), and the brain was harvested later. Homogenate of the brain was then inoculated i.c. into another macaque. Failing to obtain neurotropic virus after two passages, we then used infectious bone marrow cells (BMC) as inoculum, and this was followed by use of infectious brain homogenate.

Macaques. Yearling rhesus macaques were procured from the breeding colony of this institution. All animals were seronegative to SIVmac251 and SIVmac239. Each animal was sedated with ketamine and anesthetized with halothane, and 100 μl of virus or tissue material was injected into each cerebral hemisphere. This procedure had no clinical side effects. Animals were euthanized by exsanguination under surgical anesthesia.

Tissues. Tissues were harvested by using sterile technique and observing appropriate biohazard precautions and evaluated for virus content by multiple procedures as described elsewhere (31). Portions of tissues were fixed in 10% formalin for histological analysis and in situ hybridization, and others were frozen for polymerase chain reaction analysis. Other portions of tissues were homogenized in freezing mortars into cell extracts (10% wt/vol) in RPMI–5% fetal bovine serum. These suspensions were evaluated for cell-free infectivity. Other portions of tissue were dissociated to liberate single cells. These cells were used in infectious center assays or cultivated in special media that induced activation of resting lymphocytes (LAM [RPMI, 10% fetal bovine serum, phytohemaglutinin followed by 50 U of recombinant interleukin 2]) or differentiation of monocytes to macrophages (monocyte differentiation medium [MDM] [RPMI, 10% human AB serum, macrophage colony-stimulating factor {CSF}, granulocyte/macrophage-CSF]) as described earlier (31). Use of medium that selected for one or the other process provided a simple mechanism both for identifying latently infected cells and for determining the future tropism of such latent viral genomes. Thus, accumulation of virus particles in LAM indicated the presence of latently infected lymphocytes in tissues. Similarly, accumulation of virus in MDM indicated that latently infected monocyte-macrophages were present in tissues.

Cell-free virus assays in lymphocytes and macrophages. Virus infectivity in plasma and tissue homogenates was determined by inoculating 10-fold dilutions of samples into cultures of C8166 cells (in which both lymphocytetropic and macrophagetropic viruses replicated) and blood-derived macaque macrophages cultivated in MDM (in which only macrophagetropic viruses replicated). The C8166 cells were cultivated in RPMI–10% fetal bovine serum and examined for development of fusion 10 days later. The macrophage cultures were maintained in MDM, and supernatant fluids were examined 2 weeks later for infectivity (cytopathicity in C8166 cells).

Polymerase chain reaction. The polymerase chain reaction was used to detect viral DNA in brain cells. DNA was extracted with phenol (2×) and phenol-chloroform (1×), ethanol precipitated, and suspended in distilled H_2O. Reac-

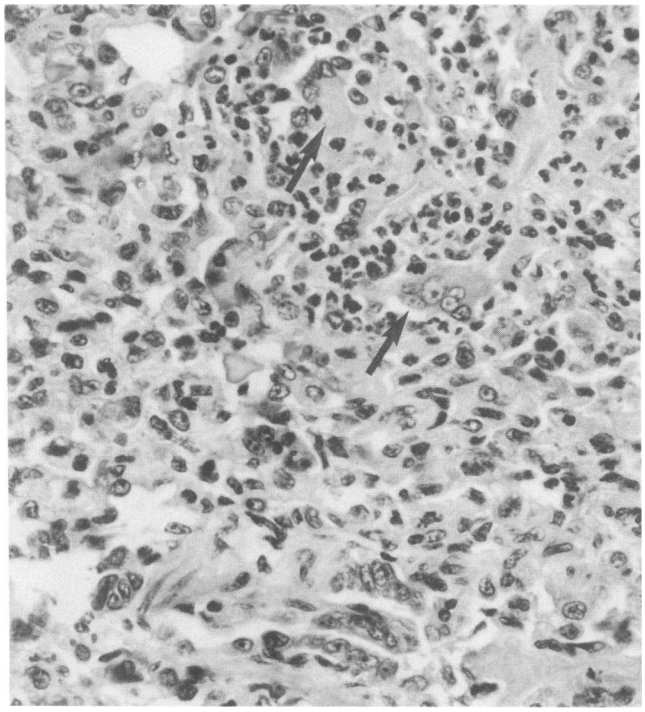

FIG. 2. Section of lung from macaque 10C showing consolidation and several multinucleated giant cells (arrows) in the interstitium. Hematoxylin and eosin; magnification, ×200.

tions were carried out using 1.0 μg of DNA per 100 μl of standard reaction mixture as previously described (3). The primers used were 5′-AAGCTTGGATCCGCATGCTAT AACACATGCTATTGT-3′ and 5′-AAGCTTGAATTCGGA GGTTCTTTGTTCCCCAGACGG-3′, which are complementary to bases 6446 to 6469 and 8379 to 8402 of SIVmac239, respectively (13). All primers have 12 nonhomologous bases at the 5′ end. To increase sensitivity, 5 μl of the first polymerase chain reaction product was then used as template for second amplification, using the conditions described above with the primers 5′-AAGCTTGGATCCGG CTTGGGGATATGTTATGAGCAA-3′ and 5′-AAGCTTG AATTCAAGTACTTCTCGATGGCAGTGACC-3′, which are complementary to bases 6512 to 6535 and 8348 to 8371. Samples were subjected to another 35 cycles as described above. Control reactions with no DNA as a negative control and cloned SIV DNA and DNA from R71 brain (Fig. 1; see Results below) as positive controls were done in parallel.

Combined immunohistochemistry and ISH. Tissue sections 6 μm thick were deparaffinized and stained histochemically by the avidin-biotin complex technique with biotinylated *Ricinus communis* agglutinin-I prior to in situ hybridization (ISH) as previously described (4). The stained tissues were then processed for ISH as described previously (4, 18). Full-length SIVmac239 DNA and an irrelevant probe were radiolabeled by nick translation using [^{35}S]dATP and [^{35}S]dCTP and used for the study (22, 34).

RESULTS

Neuroadaptation of SIVmac239. Initial attempts to neuroadapt the virus were made by using cell-free virus to inoculate two macaques (5C and 2C) (Fig. 1). Examination of tissues from both animals showed that virus-infected cells were present in bone marrow, spleen, and in peripheral blood mononuclear cells and that the cells produced virus when cultivated in LAM (31). There was no evidence from procedures to detect histological abnormalities, viral infectivity, viral RNA, and viral DNA of infection in the brain. Similarly, no virus was recovered from lung cells, macrophages derived from the lung tissue, BMC, or splenocytes cultivated in MDM. Since i.c. inoculated material usually spills over into the bloodstream (12, 25) and since the virus was strongly lymphocytetropic, infection in nonneural tissues was explained by virus infection and replication in lymphocytes in lymphoid tissues. Since the brain and lung lack such cells, no infection occurred in these organs.

Because of the inability of SIVmac239 to cause infection in brain, animals were subsequently inoculated with infectious BMC; viral RNA was observed by ISH in cells resembling macrophage precursors in the BMC (15, 32a, 34). Brain and infectious BMC of macaque 2C were mixed and inoculated i.c. into macaque 10C. This animal became acutely ill 83 days later and died overnight. Studies on tissue sections showed severe interstitial pneumonia with multinucleated giant cells (Fig. 2). ISH and immunohistochemical staining revealed viral RNA and SIV antigens in macrophages. Presence of macrophagetropic virus in tissues was also confirmed by detection of virus (10^3 $TCID_{50}$) in macrophage cultures inoculated with the BMC. However, despite infection in macrophages in the lungs, the brain showed no evidence of infection. No histological lesions were seen except for a local area of inflammation at the inoculation site, (Fig. 3) and, similar to brains from macaques 5C and 2C, this brain lacked viral RNA by ISH and viral DNA by polymerase chain reaction. Thus, infectious BMC of macaque 2C

FIG. 3. Section of brain from macaque 10C showing scarified site (arrowheads) where inoculum from 2C was deposited. Black indicates India ink added to inoculum. Cells in this site were negative for viral analysis and RNA. Hematoxylin and eosin; magnification, ×100.

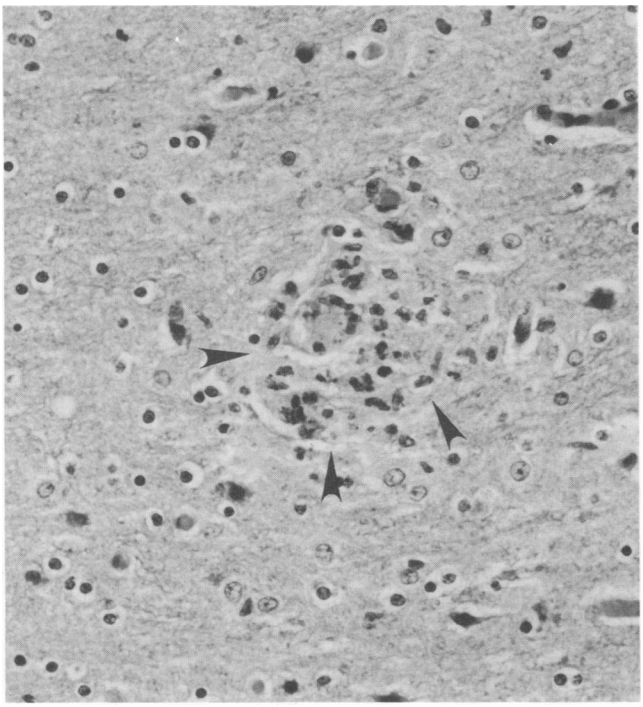

FIG. 4. Section of brain of macaque 9D showing a focal "gliotic" nodule (arrowheads) containing mononuclear and multinucleated giant cells. Hematoxylin and eosin; magnification, ×200.

administered i.c. to macaque 10C initiated infection in lymphoid tissues and lung. The inoculum probably did not contain a neurotropic phenotype. However, a macrophagetropic variant(s) of the parental virus developed in macaque 10C and caused widespread infection in macrophage populations in nonneural tissues, including the lungs. Despite this, the virus or virus-infected monocyte-macrophages did not cross the blood-brain barrier to infect microglia in the brain.

Since BMC from macaque 10C were infected with macrophagetropic virus, we used these cells to inoculate two new macaques, 9D and R71, by the i.c. route. Both animals became viremic within 10 days, with infectious plasma titers of 5×10^2 $TCID_{50}$/ml in C8166 cells. Macaque 9D died 173 days postinoculation after 2 weeks of inactivity and anorexia, and macaque R71 was euthanized 164 days postinoculation after a 3-week bout of gradual but progressive weight loss, weakness, inactivity, and ataxia.

Both animals had severe encephalitic and pneumonic lesions. Multifocal aggregates of mononuclear and occasionally multinucleated cells and a moderate number of perivascular cuffs were scattered throughout the neuropil. Lectin histochemistry demonstrated that the majority of the cells in these foci and in the perivascular cuffs were macrophages and that the giant cells were also of macrophage origin (Fig. 4 and 5). Viral RNA and antigen were present mainly in these macrophages. The lungs of both animals had focal consolidation, with evidence of cytomegalovirus infection in 9D and *Pneumocystis carinii* in R71. Several multinucleated giant cells (Fig. 6) were present in the tissue. These cells were identified immunocytochemically as macrophages, and they contained SIV antigen.

Infectivity assays of plasma and homogenates of spleen and brain of macaque 9D showed titers of 10^2, 10^4, and 10^5 $TCID_{50}$, respectively, in C8166 cultures. As shown in Fig. 7 and 8, whereas the brain virus was highly infectious for lymphocytes and macrophages, virus from plasma and the spleen were mainly lymphocytetropic. Thus, macrophagetropic and lymphocytetropic viruses were present in the same animal although located in different tissues.

Examination of the tissues of R71 showed that cell-free homogenates of spleen and lymph nodes had titers of 10^{-1} $TCID_{50}$/ml in C8166 cells but were not infectious for macrophages. Macrophage cultures derived from spleen and lymph nodes also had low virus titers. In contrast, cell-free homogenates of brain and lung had infectivity titers of 5×10^5 and 5×10^4 $TCID_{50}$/mL, respectively, in both C8166 and macrophage cultures. Further, supernatant fluids of macrophage cultures derived by explanation of fragments of these tissues had titers in excess of 10^4 $TCID_{50}$/ml. Thus, in both animals, macrophages in the lung and brain but not those in the spleen and lymph nodes were the main sites of virus replication. The spleen had mainly lymphocytetropic virus, and this was reflected in the plasma, from which only lymphocytetropic virus was obtained.

We next asked whether the neurovirulent virus had a specific tropism for the brain and whether the virus had neuroinvasive properties after inoculation into a nonneural site. Macaque R71 brain homogenate, containing approximately 10^5 $TCID_{50}$/ml, was used; 0.5 ml was inoculated into the bone marrow of two macaques, 16D and 22D, and 0.1 ml was inoculated i.c. into another animal, 17E. All three animals became viremic by 10 days postinoculation, and the viremia persisted during the following 3 months. Plasma titers fluctuated between 5 and 50 $TCID_{50}$/ml, and infectious center assays on peripheral blood mononuclear cells

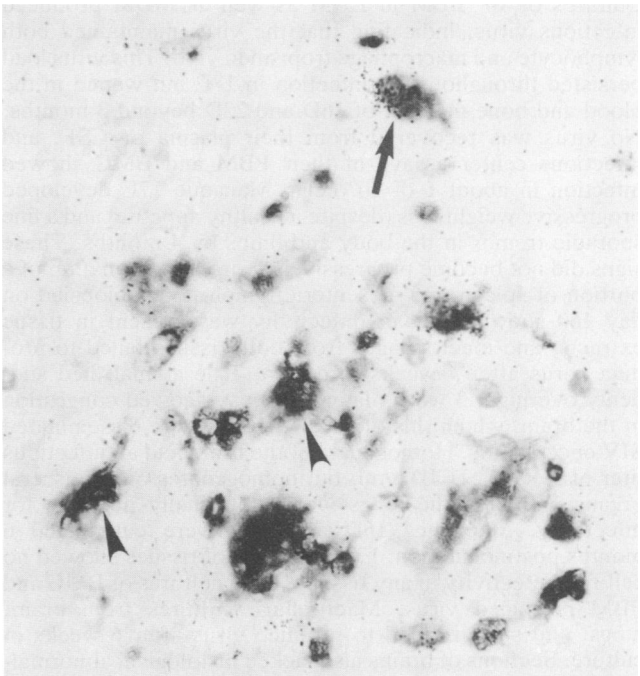

FIG. 5. Section of brain from macaque 9D. The section was stained with RCA-I, a lectin which detects microglia (positive cells indicated by arrowheads), and then processed for ISH with ^{35}S-labeled SIVmac239 DNA (indicated by grains over cells). Arrow indicates a cell with colocalization of the two labels. Magnification, ×350.

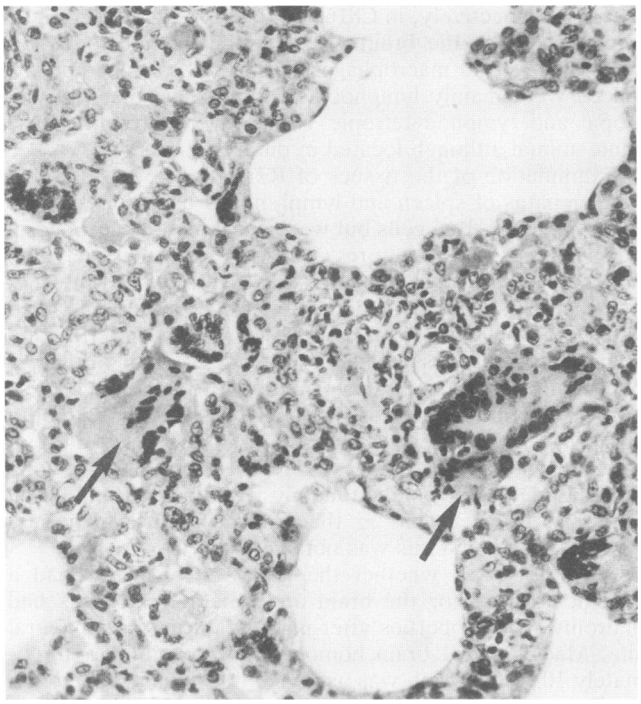

FIG. 6. Section of lung from macaque R71 showing multiple large multinucleated giant cells (arrows) in the interstitial spaces and alveoli. Hematoxylin and eosin; magnification, ×200.

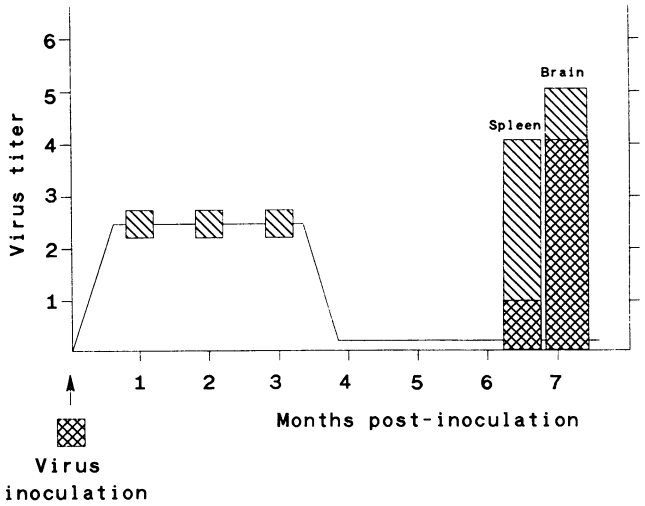

FIG. 7. Tropism of virus for lymphocytes or macrophages in different tissues of macaque 9D. Virus in the inoculum was tropic for both cell types. Plasma sampled at months 1, 2, and 3 had a titer of 10^2 $TCID_{50}$ in lymphocytes but was not infectious for macrophages; spleen homogenate had a titer of 10^4 in lymphocytes and 10^1 in macrophages; brain homogenate had a titer of 10^5 in lymphocytes and 10^4 in macrophages. ▧, lymphotropic virus; ▩, macrophage-tropic virus.

(PBMC) and BMC showed that between 1 in 10^3 and 1 in 10^4 cells were infected. Cells in the CSF were also infectious. Cultures of 10^6 PBM in LAM as well as MDM produced infectious virus, indicating that the virus maintained both lymphocyte and macrophage tropism in vivo. This virus load persisted throughout the infection in 17E but waned in the blood and bone marrow of 16D and 22D beyond 3 months. No virus was recovered from their plasma or CSF, and infectious center assays of their PBM and BMC showed infection in about 1 of 10^6 cells. Macaque 17E developed progressive weight loss (despite a healthy appetite) and a fine sporadic tremor in the body and limbs by 4 months. These signs did not become progressively worse. Examination of a portion of spleen and mesenteric lymph nodes biopsied on day 160 showed that no infectivity was present in tissue extracts, and macrophages from both tissues failed to produce virus after 4 weeks in culture. The animal died suddenly overnight 3 weeks later. Autopsy showed congestion in the brain, which, histologically, had severe disseminated SIV encephalitis. Homogenate of the brain had an infectious titer of 5×10^4 $TCID_{50}$/ml, but homogenates of all visceral organs including the lungs were uniformally negative for infectivity. Macaques 16D and 22D were euthanized 6 months postinoculation. Examinations of tissues showed no cell-free infectivity in any tissues. Only cultures of BMC and PBM produced virus. Macrophage cultures from brain, lungs, and spleen failed to produce virus after 6 weeks in culture. Sections of brains also lacked histological abnormalities and viral RNA. This experiment thus showed that the neurotropic virus SIVmac239-R71-BR maintained its macrophage tropism. In in vitro showed greater selectivity by replicating vivo, it replicated productively only in microglia. When inoculated peripherally, it infected cells in the bone marrow but not macrophages in any visceral organs. Further, the brains in these two animals did not become infected either by infected cells migrating from the bone marrow or by virus-infected cells that had been present in the CSF earlier.

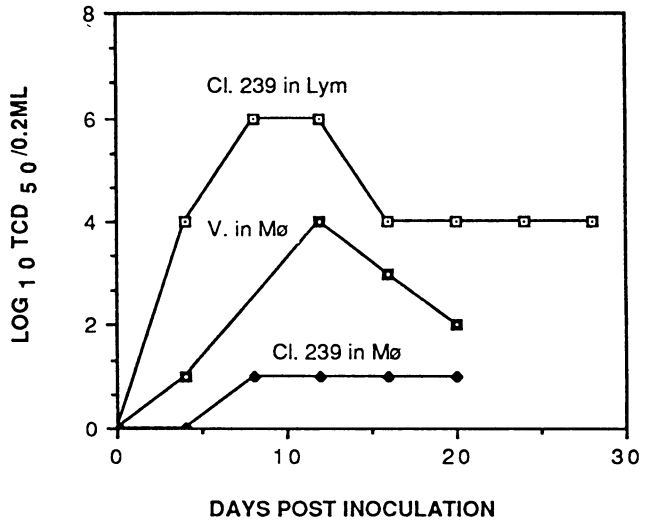

FIG. 8. Growth curves comparing the tropism of parental virus (molecularly cloned SIVmac239) with that of brain virus from macaque 9D. Both viruses were inoculated into C8166 and primary macrophage cultures, and supernatant fluids were examined for infectivity at various times afterwards. Macrophage cultures inoculated with SIVmac239 had negligible infectivity in comparison to lymphocyte (Lym) cultures, which had a titer of 5×10^6/ml. The variant (V.) virus in 9D brain homogenate replicated productivity in macrophage (Mø) cultures, reaching a titer of 5×10^4/ml by day 13.

DISCUSSION

We report here on the derivation of a neurovirulent variant of molecularly cloned, nonneurotropic SIVmac239 by using infectious BMC instead of virus as inoculum. Previous studies had shown that virus 239 is mainly lymphocytetropic and replicates extensively in lymphatic tissues of infected macaques (13, 31). In experiments described here, this virus maintained its lymphotropism but failed to infect any cells in the brain after i.c. inoculation into two macaques. In contrast, infection in the brain was accomplished readily after i.c. inoculation of macaques with infected BMC.

Two serial passages using the BMC-to-brain protocol yielded a highly neurovirulent virus in macaque R71. The variant virus SIVmac239-R71-BR replicated productively and preferentially in brain and was located mainly in microglial cells. Failure of this virus to replicate productively in other tissues suggests that this virus had an affinity mainly for microglia. This was reflected in part in vitro, where the virus replicated productively in macrophages (unlike parental virus 239). However, it is apparent that the cultured macrophages could not distinguish among viruses that had tropisms for specific populations of macrophages. Reconstruction of events in the neuroadaptation process suggests that acquisition of macrophage tropism by the virus is a prerequisite for its neurotropism. This event may have occurred by mutation of the virus and selection in specific BMC. Further selection for microglia-specific virus occurred after infectious BMC were inoculated into the neuropil.

Despite the highly neurovirulent nature of R71-BR virus, animals inoculated parenterally with this agent did not develop infection in the central nervous system despite persistent infection in monocyte-macrophages in blood and bone marrow and even spread of virus-infected cells to the CSF. This suggested that although the virus entered the CSF, it was still poorly neuroinvasive. Similar poor neuroinvasiveness has been observed in highly neurovirulent strains of ovine lentiviruses (27, 33). Whether this phenomenon is relevant in HIV infection is not known. Although HIV invades the CSF early in infection (28), it is still open to speculation whether the virus gains access to the brain at this time.

The tissue selection of specific viral phenotypes in the tissues macaques of 9D, R71, and 17E was unexpected. Whereas the brain and lung selected for macrophagetropic agents, the spleen selected mainly for a lymphocytetropic virus. The finding that the virus in plasma was also lymphocytetropic added further support to the suggestion that viremia virus was derived mainly from spleen and lymph nodes (31). Since the brain and lungs are rich in macrophages and the spleen and lymph nodes are rich in CD4+ lymphocytes, cells in these tissues probably provided a strong selection system for the two viral phenotypes. Further selection of specific viruses may have occurred in different macrophage populations, because whereas cultures of the splenic macrophages produced negligible infectious virus, macrophages of the brain and lungs were highly productive. This variety of viral phenotypes may have originated among the cells of the infected BMC of macaque 10C that were used for inoculation of both 9D and R71. Since BMC include precursors of highly heterogenous leukocyte populations, different cell types could represent potential selectors for specific viral phenotypes from among the huge numbers of quasispecies of virus that arise by constant mutation (6).

In contrast to the multiplicity of phenotypes that develop in the bone marrow, the virus from brain of R71 was almost exclusively tropic for brain macrophages. This agent replicated productively only in the brain of 17E. After intravenous inoculation into two other animals (16D and 22D), it failed to invade the central nervous system. Interestingly, it also failed to replicate productively in any macrophage population in nonneural tissues. The regimen of i.c. inoculation of virus may thus have resulted in selection and amplification of a microglia-specific biological clone of this virus. A similar tissue selection of specific molecularly characterized viral phenotypes has been reported in mice persistently infected with lymphocytic choriomeningitis virus (1, 2). Equally important and more relevant to our study, a similar segregation of HIV genotypes has been observed in the brain and blood of a patient with HIV encephalopathy (17) and in the brains and spleens of children with AIDS (10). This phenomenon of tissue selection may thus be common among viruses that cause persistent infections and may provide the basis for appearance of new clinical manifestations of disease caused by constant mutation and selection of new viral phenotypes. Neurotropic viruses may be one of such phenotypes.

ACKNOWLEDGMENTS

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