Journal of NeuroVirology (1998) 4, 269–280

http://www.jneurovirol.com

Efficient infection of brain microvascular endothelial cells by an *in vivo*-selected neuroinvasive SIV_{mac} variant

Lisa I Strelow[1], Debbie D Watry[2], Howard S Fox[2] and Jay A Nelson[1]

[1]*Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201;*
[2]*Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037 USA*

A neuroinvasive/neuropathogenic SIV variant termed SIV_{mac}182 was previously isolated and characterized (Watry *et al*, 1994). This neuroinvasive strain was derived from the uncloned strain SIV_{mac}251 through serial animal passage of infected microglia, unlike previously reported neurovirulent strains. Importantly, the virus described here was isolated from a strain which already demonstrates limited neuroinvasiveness *in vivo*, through a route of inoculation which exerts selective pressure for variants in the periphery that can naturally cross the blood-brain barrier and gain access to the brain. Examination of animal tissues indicated that the neuroinvasive strain was capable of replicating in brain microvascular endothelial cells (BMEC). Therefore, we developed an *in vitro* model of BMEC infection in which to examine mechanisms of virus neuroinvasiveness and neuropathogenicity as well as to address mechanisms of HIV-induced dementia. Results obtained with this *in vitro* system indicate that growth in BMEC may predict neuroinvasiveness *in vivo*, and furthermore, that brain passage of virus results in the generation of neuroinvasive strains which demonstrate an increased efficiency of BMEC infection *in vitro*.

Keywords: AIDS dementia; SIV; endothelial cells

Introduction

Infection with human immunodeficiency virus type 1 (HIV-1) frequently results in neurological dysfunction throughout the disease course. A syndrome of cognitive and motor dysfunction has been recognized and termed the AIDS dementia complex (ADC) (Spencer and Price, 1992; Navia *et al*, 1986a). ADC occurs in approximately 15–30% of late disease stage patients (McArthur *et al*, 1993) and manifests as a subcortical dementia with pyschomotor retardation, cognitive impairment and behavioral changes (Sharer, 1992; Power *et al*, 1993). Histopathologically, ADC patients at autopsy present with diffuse pallor of the white matter, astrogliosis, multinucleated giant cells, microglial nodules, vacuolar myelopathy, increased numbers of perivascular macrophages and possible loss of oligodendrocytes and neurons (Navia *et al*, 1986b; Sharer, 1992; Budka *et al*, 1991). ADC is a separable pathological entity from HIV encephalitis (HIVE) in that half of the patients presenting with ADC do not exhibit neuropathological evidence of HIVE (Glass *et al*, 1993; Sharer, 1992; Navia *et al*, 1986b, Budka, 1991). Thus, while HIVE appears to be primarily an inflammatory process marked by prominent inflammatory infiltrates, the pathology of ADC can be more subtle. Despite numerous studies, the contribution of viral and host factors in CNS disease is not clearly understood.

In both HIVE and ADC, the predominantly infected cell type is the macrophage/microglial cell (Takahashi *et al*, 1996; Wiley *et al*, 1986; Budka *et al*, 1991; Vazeux *et al*, 1987), with fewer but significant numbers of capillary endothelial cells (Wiley *et al*, 1986; Moses *et al*, 1996a; Koenig *et al*, 1986; Gabuzda *et al*, 1986; Ward *et al*, 1987) and possibly astrocytes (Tornatore *et al*, 1994; Saito *et al*, 1994) also demonstrating evidence of *in vivo* infection. Neurons do not appear to be infected in significant numbers. The presence of virus in macrophage/microglial populations and the perivascular localization of HIV-1 antigens in the CNS lead to the hypothesis that virus may gain access to

Correspondence: J Nelson
Received 2 February 1998; accepted; 13 February 1998

the CNS via trafficking of infected macrophages across the blood-brain barrier (BBB). Although HIV-1 is detectable in the CNS of most AIDS patients, the amount of detectable virus does not correlate with levels of either brain pathology or dementia (Lazarini *et al*, 1997; Glass *et al*, 1993), suggesting that the viral mechanisms underlying dementia are indirect. One such mechanism for indirect effects of HIV-1 infection involves perturbations of BBB function. The BBB is a selectively permeable barrier between the systemic circulation and the CNS, consisting of endothelial cells lining cerebral microvessels and in contact with astrocytes at the abluminal surface of the capillary. The endothelial cells lining cerebral microvessels and forming BBB are characterized by limited vesicular transport, limited pinocytotic vesicles, selective active transport mechanisms and the presence of continuous tight junctions (Joo, 1985; Turner *et al*, 1987; Goldstein and Lorris Betz, 1983). Serum protein leakage into the brains of AIDS patients but not control brains has also been reported (Rhodes, 1991; Singer *et al*, 1994; Petito and Cash, 1992), indicating the presence of BBB dysfunction in HIV-1-infected individuals. In the study by Petito and Cash (1992), half of all AIDS brains had evidence of diffuse serum protein leakage into brain in the absence of detectable encephalitis. Additionally, increased cerebrospinal fluid (CSF)-serum albumin ratios, indicative of BBB damage, occur in HIV infection and are much higher in ADC patients (Power *et al*, 1993). HIV-1 infection of capillary endothelial cells has been demonstrated both *in vivo* and *in vitro* (Wiley *et al*, 1986; Moses *et al*, 1996a; Koenig *et al*, 1986; Gabuzda *et al*, 1986; Ward *et al*, 1987; Moses *et al*, 1993), and thus may represent an alternate mechanism by which virus is able to cross the BBB and/or contribute to manifestations of ADC.

Simian immunodeficiency virus (SIV) infection of macaques induces simian AIDS and CNS pathology which parallels that found in human AIDS, thereby providing a suitable model for studies of HIV-induced CNS dysfunction (Desrosier 1990; Chakrabarti *et al*, 1991; Lackner *et al*, 1991; Sharer *et al*, 1991; Simon *et al*, 1992). As with HIV, SIV-infected cells of the CNS are predominantly infiltrating macrophages and microglia (Simon *et al*, 1992). In addition, SIV infects brain capillary endothelial cells *in vivo* and *in vitro* (Flaherty *et al*, 1997; Mankowski *et al*, 1994, this study). SIV-infected macaques demonstrate functional CNS deficiencies, including cognitive and motor dysfunctions and behavioral disturbances, much like human ADC patients (Prospero-Garcia *et al*, 1996; Murray *et al*, 1992). Abnormalities in BBB function have been described in SIV-infected macaques (Prospero-Garcia *et al*, 1996; Smith *et al*, 1994), a finding which further confirms the utility of the SIV model for studies of CNS disease. Furthermore, macaques can be infected with defined, molecular clones of SIV and examined at various times post-inoculation in order to define early viral events.

Macaques inoculated with the prototypical, lymphocyte-tropic molecular clone $SIV_{mac}239$ develop immunosuppression and AIDS but do not exhibit neurologic disease (Stephens *et al*, 1995; Sharma *et al*, 1992a,b). Although infectious virus cannot be cultured from the brains of $SIV_{mac}239$-infected macaques, Stephens *et al* (1995) and Zhu *et al* (1995) have reported that $SIV_{mac}239$ can cause a persistent infection of the CNS, as evidenced by isolation of viral DNA. Furthermore, their studies indicate that the viral sequences obtained from brain are those of the original lymphocyte-tropic strain. In the cases where neurologic disease has resulted following infection with $SIV_{mac}239$, however, infectious virus isolated from brains had evolved macrophage tropism and therefore differed from the input lymphocyte-tropic SIV (Sharma *et al*, 1992b; Desrosiers *et al*, 1991; Anderson *et al*, 1993). Results from several studies suggest that macrophage tropism is a prerequisite for neurovirulence, or the ability of virus to replicate in brain (Sharma *et al*, 1992b; Spencer and Price, 1992). Recent studies by Flaherty *et al* (1997) and Mankowski *et al* (1997) however, suggest that macrophage tropism alone is not sufficient for neurovirulence, in that a recombinant virus, SIV/17E-Cl, which replicates in macrophages but not in brain capillary endothelial cells does not cause neurological disease. Neuroinvasiveness has been defined as the ability of virus to gain access to the CNS via trafficking of infected cells. Here we define neuroinvasiveness as the ability of virus in the peripheral blood to cross the BBB and get into the brain parenchyma, regardless of mechanism. Thus, inflammatory cells are an important factor in the pathogenesis of HIVE and certainly contribute to the viral load in brain. These inflammatory cells may not be as important for the pathogenesis of AIDS dementia, however, as there are alternative mechanisms for virus to gain CNS access, such as through interactions with the endothelial cells lining cerebral microvessels (Moses and Nelson, 1994). In addition, neurovirulence refers to the ability of a virus to replicate in and cause alterations to the CNS. By the latter criterion, $SIV_{mac}239$ is not neurovirulent, since virus inoculated directly into brains failed to replicate (Sharma *et al*, 1992a). Although $SIV_{mac}239$ can be isolated from cerebrospinal fluid (CSF) as early as one-week post-inoculation (Sharma *et al*, 1992a) the presence of virus in CSF does not necessarily indicate brain infection (Sharma *et al*, 1992a,b).

In contrast, infection with the uncloned stock of $SIV_{mac}251$ results in a brain infection accompanied by gliosis as early as 7 days post-inoculation (Chakrabarti *et al*, 1991). *In situ* hybridization for viral *nef* sequences localized $SIV_{mac}251$ in perivascular regions in the brain at early time points.

Additionally, neuropathological changes worsened with disease progression. Another study detected virus-specific CTL as well as virus in CSF within 1 week of inoculation with uncloned $SIV_{mac}251$ (von Herrath *et al*, 1995). This observation is in stark contrast to the molecular clone of $SIV_{mac}251$, for which infection resulted in significantly reduced mortality compared to the strain from which it was derived (Edmonson *et al*, 1998; Daniel *et al*, 1985, 1987; Letvin *et al*, 1985). The reasons for the attentuated pathogenicity of the molecular clone are unknown.

Several groups have attempted to 'neuroadapt' non-neuroinvasive strains of SIV through serial animal passage of infected cells (Sharma *et al*, 1992b; Mankowski *et al*, 1994; 1997; Flaherty *et al*, 1997; Watry *et al*, 1995; Lane *et al*, 1995). In a set of studies by Sharma *et al* (1992b), the parental strain $SIV_{mac}239$ was passaged through animals by intracerebral inoculation of both brain and bone marrow homogenates. The strain which resulted after four animal passages was termed SIV/17E-Br. This neuroadapted strain readily infected the brain, where infection was localized mainly to microglial cells. An animal dying of SIV/17E-Br infection demonstrated severe disseminated SIV encephalitis. Thus, serial passage through macaque brain resulted in a neuroadapted SIV. This strain also productively replicated in brain microvascular endothelial cells (BMEC) both *in vivo* and *in vitro*, whereas the parental $SIV_{mac}239$ failed to replicate in BMEC (Mankowski *et al*, 1994). Further studies with the SIV/17E-Br strain led to the derivation of two recombinant molecular clones, SIV/17E-Cl and SIV/17E-Fr (Flaherty *et al*, 1997; Mankowski *et al*, 1997). SIV/17E-Cl contains the *env* gp120 and a portion of gp41 from the SIV/17E-Br neurovirulent strain in the background of non-neurovirulent $SIV_{mac}239$. Although this virus replicated in macrophages, this strain did not productively infect BMEC *in vitro*, nor did inoculation into macaques result in brain disease, suggesting that macrophage tropism alone is not sufficient for neurovirulence (Mankowski *et al*, 1997; Flaherty *et al*, 1997). SIV/17E-Fr contains the entire *env* and *nef* genes as well as the 3′ long terminal repeat (LTR) from the neurovirulent strain SIV/17E-Br in the background of $SIV_{mac}239$. This virus replicated in both macrophages and BMEC *in vitro*, and caused neurologic disease in the majority of inoculated macaques (Mankowski *et al*, 1997; Flaherty *et al*, 1997).

Since most neuroinvasive strains of SIV also demonstrate macrophage tropism, an enriched population of infected macrophages/microglia was isolated and used to inoculate animals in order to neuroadapt uncloned $SIV_{mac}251$ (Watry *et al*, 1995; Lane *et al*, 1995). Two rounds of intravenous animal passage yielded a neuroinvasive and neurovirulent strain, $SIV_{mac}182$. In this study, one animal receiving first passage virus and all three animals receiving second passage virus developed significant neuropathology (Watry *et al*, 1995). As inoculations were done intravenously, the ability of the virus to cause brain pathology indicates that it is both neuroinvasive and neurovirulent. In addition, the results demonstrate that neuropathogenic virus partitioned to the CNS during a natural SIV infection. An analysis of *env* sequences from the serially passaged, microglia-associated SIV indicated that there was an enrichment for *env* quasispecies correlating with neuropathology (Lane *et al*, 1995). The genetic changes following animal passage occured predominantly in the V1 and V4 regions of *env*, suggesting that these sequences may contribute to neurovirulence.

In the present study, we define a system for examining determinants of neuroinvasiveness as relates to potential mechanisms for AIDS dementia. Our model system involves *in vitro* infection of brain microvascular endothelial cells (BMEC) by distinct SIV clones with defined tropisms and phenotypes. Thus, $SIV_{mac}239$ is not neurovirulent, nor does it infect simian BMEC. $SIV_{mac}251$ molecular clone has limited virulence in inoculated animals. The $SIV_{mac}251$ clone does infect simian BMEC *in vitro*. Finally, the uncloned strain $SIV_{mac}182$ is both neuroinvasive and neurovirulent and, like cloned $SIV_{mac}182$, infects BMEC *in vitro*. In addition, we demonstrate that animal passage of uncloned $SIV_{mac}251$ selects for a variant, $SIV_{mac}182$, which is neuroinvasive, neurovirulent and able to efficiently infect BMEC.

Results

In situ *hybridization*

In an attempt to derive a virus capable of inducing CNS disease, uncloned $SIV_{mac}251$ was serially animal passaged through naturally infected microglia (Watry *et al*, 1995). To determine if this virus strain was capable of being neuroinvasive and neurovirulent *in vivo*, a sensitive *in situ* hybridization for viral sequences was performed on brain sections of infected animal 182 (from whose brain the cell-free stock of $SIV_{mac}182$ was derived). *In situ* hybridization for SIV *nef* sequences revealed specific signals over cells lining small and medium sized vessels in the brain of SIV-infected macaque 182 (Figure 1A and B). These cells had the morphological appearance of endothelial cells. In addition, positive hybridization signals were observed over microglial nodules, multinucleate giant cells and perivascular macrophages. These results demonstrate that microglia-passaged SIV is able to productively infect brain tissue *in vivo*, confirming the neuroinvasiveness and neurovirulence of this virus. Although the identification of the SIV-positive cells cannot be unequivocably confirmed as endothelial cells without double label analysis,

the perivascular localization of many of the signals suggests that BMEC are a target for microglia-passaged SIV infection *in vivo*.

Isolation and characterization of simian brain microvascular endothelial cells (BMEC)

The *in situ* results indicated that microglia-passaged SIV was neuroinvasive and neuropathogenic. Therefore, to determine if the neuroinvasive SIV_{mac} 182 (an SIV strain derived from the brain of animal 182) exhibited an unusal ability to infect brain microvascular endothelial cells (BMEC) *in vitro*, primary simian BMEC cultures were established from simian retrovirus type D-, SIV-, herpes B- and simian foamy virus-negative rhesus macaque brain tissue as described in Materials and methods. All macaque tissues were routinely screened for the presence of simian foamy virus (SFV) by PCR for *env* sequences as well as by coculture on permissive fibroblasts. Macaque tissues were not used in studies until their SFV-negativity was confirmed, since infection with SFV results in syncytium-induction and cytopathic effect on simian BMEC in culture (our unpublished observations) and since infected tissues are capable of transmitting this virus. After several days in culture, individual BMEC micro-colonies were subcultured using cloning rings and amplified prior to cryopreservation at passage three (Figure 2A). BMEC were identified by the presence of the endothelial cell-specific marker von Willebrand factor VIII-related antigen (vWF) (Figure 2B) as well as by positive immunoreactivity for endoglin (TGFβ receptor). Initial cultures demonstrated an endothelial cell purity of $>95\%$. The rabbit anti-human vWF antibody did cross react with simian cells, although the staining was not as intense as that seen for human BMEC. Purity of cultures was also established by the lack of immunoreactivity for smooth muscle actin, glial fibrillary acidic protein (GFAP; astrocytes), and TE-7 (mesenchymal cells and fibroblasts). The isolation procedure involves selective adherence of the brain homogenate to glass beads, since most brain cell types other than capillaries will not adhere to glass. Cells were passaged three times prior to cryopreservation in order to preclude contamination with macrophages and peripheral blood mononuclear cells, which will not survive repeated passaging.

Viral growth curves

BMEC cultures described above were utilized to determine the ability of the SIV strains used in this study to infect BMEC *in vitro*. Cell-free virus derived from culture of the microglia of animal 182 (SIV_{mac}182 uncloned strain) could infect BMEC *in vitro*, as assayed by PCR for viral *env* sequences at 9 days p.i. (data not shown). To verify that the molecular clones SIV_{mac}251 and SIV_{mac}182 clone 1100 (derived from the brain of animal 182) infected BMEC, simian BMEC at low passage were inoculated and assayed daily for production of viral p27 by a commercial antigen capture ELISA (Coulter) (Figure 3). Levels of supernatant p27 were measured for at least 14 days post-inoculation (p.i.). Cells were washed daily to ensure measurement of daily production of supernatant p27. Residual input inoculum of SIV_{mac}251 was cleared by day 4 p.i. (Figure 3A). Virus production then continued to increase from days 6 through 14 p.i. SIV_{mac}182 clone 1100 infected BMEC with faster kinetics than SIV_{mac}251 (Figure 3B). Thus, residual input inoculum was cleared by day 2 p.i., and virus production increased dramatically from day 3 onward. The yield of supernatant p27 from SIV_{mac}182 clone 1100-infected BMEC was up to 100-fold higher than for SIV_{mac}251 at all time points tested, indicating that SIV_{mac}182 clone 1100 was able to productively infect BMEC cultures much more efficiently. Ad-

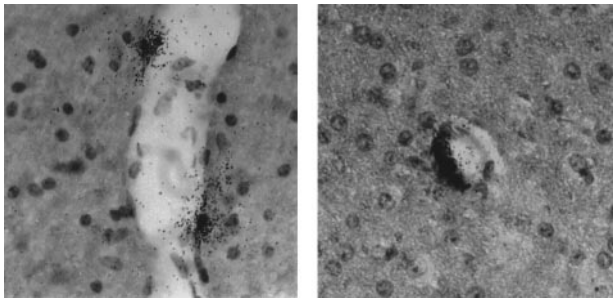

Figure 1 *In situ* hybridization for *nef*. Macaque 182 was sacrificed at 69 days post-inoculation due to rapidly progressing disease. Brain sections were fixed and paraffin embedded prior to *in situ* hybridization using an antisense probe for SIV *nef*. Note the silver grains deposited over cells lining brain microvessels. Positive hybridization signals were also observed over microglial nodules, multinucleate giant cells and perivascular macrophages. Magnification $\times 400$.

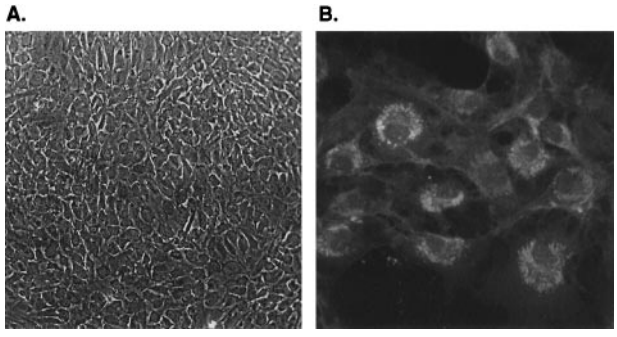

Figure 2 Isolation and characterization of simian BMEC. BMEC were isolated from simian retrovirus type D-, SIV-, herpes B- and simian foamy virus-negative macaques as described in Materials and methods. (**A**) Phase contrast of a confluent monolayer of passage 3 simian BMEC showing characteristic cobblestone morphology. (**B**) Indirect immunofluorescence for the endothelial cell-specific marker vWF. Primary simian BMEC were fixed and stained for vWF using a monoclonal antibody to vWF and a rhodamine-conjugated secondary antibody.

ditionally, days 9–13 p.i. represent values at the limit of the linear range for detection by the ELISA, and therefore may be higher than shown. Both SIV clones produced virus continuously through day 21 p.i. (Figure 4 and data not shown). These results demonstrate that virus derived from the brain of animal 182 is able to productively infect BMEC *in vitro*, and furthermore, that $SIV_{mac}182$ clone 1100 is able to infect BMEC with greater efficiency. This latter observation suggests that microglial passage selects for a variant which also efficiently infects BMEC. This observation has important mechanistic implications for induction of AIDS dementia. Additionally, cytopathic effects were never observed in these cultures when inoculums without simian foamy virus were used. This observation is in contrast to a previous study in which syncytial cells were routinely observed in BMEC inoculated with a different strain of SIV_{mac} (Mankowski, 1994). Cytopathic effects were never observed in any of our long-term SIV_{mac}-infected BMEC (Figure 4 and data not shown), suggesting that even neuroinvasive and neurovirulent SIV_{mac} is able to infect BMEC in a non-cytopathic manner, as would be expected for a virus which is able to cause subtle perturbations in brain function.

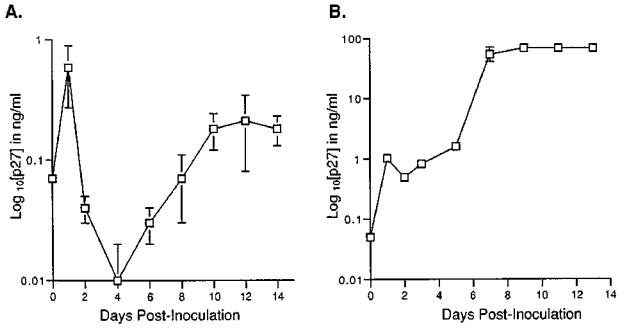

Figure 3 Kinetics of SIV p27 production in BMEC. Simian BMEC at passage 5 were inoculated with (**A**) cloned $SIV_{mac}251$ at an MOI of between 0.02 and 0.4 or (**B**) $SIV_{mac}182$ clone 1100 at an MOI of 0.03. Daily virus production was measured by ELISA for supernatant levels of p27 antigen. Each time point represents the average of six replicates for $SIV_{mac}251$ and three replicates for $SIV_{mac}182$ clone 1100. Error bars represent the standard deviation of replicates.

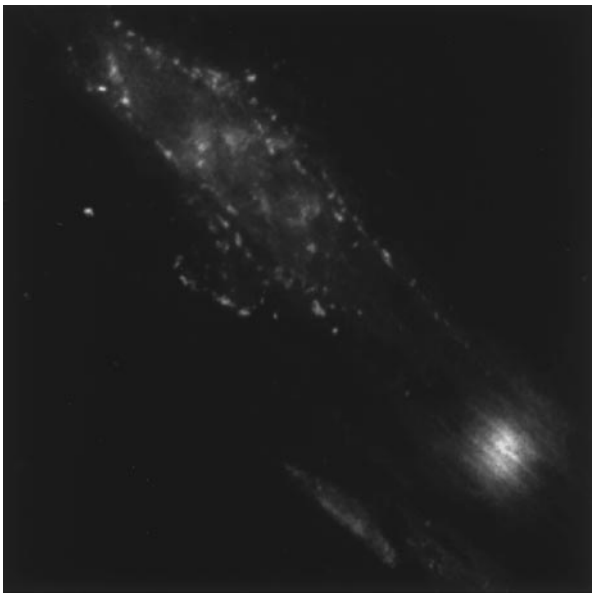

Figure 4 Immunofluorescence for SIV p27. Indirect immunofluorescence for SIV p27 using a mouse monoclonal antibody to p27 and a fluorescein-conjugated secondary antibody. Simian BMEC were inoculated with cloned $SIV_{mac}251$ at an MOI of 0.4 and stained for the presence of p27 at 21 days post-inoculation. A typical section of endothelial cell monolayer is shown, with the frequency of p27-positive cells estimated at $<5\%$.

Immunofluorescence for viral p27

Simian BMEC were inoculated with cloned SIV_{mac} 251 and assayed for the presence of virus by indirect immunofluorescence for viral p27 at various times p.i. in order to measure the number of productively-infected cells as well as to document the ability of cloned $SIV_{mac}251$ to spread in BMEC culture. Virus was undetectable at early times p.i. (days 3, 5 and 7). By day 10 p.i., however, small numbers of p27-immunoreactive cells were seen (data not shown), in good agreement with culture supernatant p27 levels. The pattern of p27 staining was seen as punctate and localized to the cytoplasm of cells (Figure 4). p27 immunoreactive cells were detectable through day 21 p.i. (Figure 4), however, the number of p27-positive cells did not increase from earlier time points. By estimating the numbers of p27 positive cells, no more than 5% of BMEC in culture were infected by cloned $SIV_{mac}251$ at any time, in spite of the levels of p27 produced in culture supernatants. This result suggests that cloned $SIV_{mac}251$ is able to generate a low-level infection of BMEC *in vitro*, and furthermore, that cloned $SIV_{mac}251$ does not appear to spread efficiently in BMEC cultures. A single time point of infection (day 10 p.i.) was measured for $SIV_{mac}182$ clone 1100 infection of BMEC. At this time, approximately 40% of cells stained for p27 (data not shown), indicating that $SIV_{mac}182$ clone 1100 is able to either infect BMEC more efficiently than cloned $SIV_{mac}251$, or that $SIV_{mac}182$ clone 1100 is able to spread in BMEC cultures.

Discussion

In this study we characterized a model for examining mechanisms of viral neuroinvasiveness as relates to potential mechanisms of AIDS dementia. Our model entails the *in vitro* infection of brain microvascular endothelial cells (BMEC) as an indication of a virus' potential to be neuroinvasive *in vivo*. Our results

show that animal passage of virus selected for a neuroinvasive and neuropathogenic strain which was able to infect BMEC efficiently. This model system will enable a dissection of some of the viral factors involved in the pathologies of AIDS dementia complex (ADC), which is separable mechanistically from HIV-induced encephalitis (HIVE).

Importantly, the virus described here was derived from an uncloned strain of SIV after only two animal passages of infected brain microglia. In a previous study by Sharma *et al* (1992b), the lymphocyte tropic clone $SIV_{mac}239$ was neuroadapted by serial animal passage of brain and bone marrow homogenates. $SIV_{mac}239$ is not neuroinavasive (Sharma *et al*, 1992b; Desrosiers *et al*, 1991; Anderson *et al*, 1993), and the derivation of the uncloned strain SIV/17E-Br reflected this restriction, since four rounds of animal passage were required to neuroadapt the parental virus (Sharma *et al*, 1992b). In addition, the use of bone marrow homogenates was introduced in order to help enrich for macrophage-tropic strains of SIV. Interestingly, bone marrow is the only source of microvascular endothelial cells other than brain which is naturally infected by HIV (Moses *et al*, 1996c). More recent studies by Flaherty *et al* (1997) and Mankowski *et al* (1997) with clones of the SIV/17E-Br stock suggest that macrophage tropism alone is not sufficient for neurovirulence. Therefore, the enrichment for macrophage-tropic virus in bone marrow may have exerted selective pressure unrelated to neuroinvasiveness. $SIV_{mac}239$ was passaged through direct intracerebral inoculation of homogenates. Peripheral inoculation of virus, as was performed for the derivation of $SIV_{mac}182$, may help to select for variants which can naturally cross the BBB and gain access to the brain. Indeed, several studies have shown that animal selection of $SIV_{mac}239$ through the intravenous route results in the generation of a population of virus that has evolved macrophage-tropism and neuroinvasiveness (Sharma *et al*, 1992b; Desrosiers *et al*, 1991; Anderson *et al*, 1993).

In contrast with a previous study (Mankowski *et al*, 1994), syncytium-induction or cytopathic effect of SIV on BMEC was not observed during long-term culture. However, SFV will induce significant cytopathic effects on simian BMEC, including syncytium formation (our results). SFV infects up to 50% of adult rhesus macaques at the Oregon Regional Primate Research Center (Michael Axthelm, personal communication and our unpublished results), and therefore represents a significant source for contaminating retroviruses in macaque tissues. Therefore the lack of an SIV-induced cytopathic effect on BMEC is significant, in that this observation suggests that SIV is able to productively infect BMEC without causing overt cell damage. We would expect a virus which infects cells of the BBB to have such a property, since limited cell damage is seen in AIDS dementia patients.

In our model system, the kinetics of virus production in BMEC are in good agreement with previously published results for BMEC infection by SIV/17E-Br *in vitro* (Mankowski *et al*, 1994). Although difficult to compare, the yields from $SIV_{mac}182$ clone 1100 appear to be much higher than those obtained for SIV/17E-Br, whereas molecular clones $SIV_{mac}251$ and SIV/17E-Fr appear to have lower yields (Flaherty *et al*, 1997; this study). These results may reflect differences between laboratories and different conditions used, and will be important to address in future studies.

The role of SIV *env* sequences in neuroinvasion and neuropathogenicity is currently under investigation (Mankowski *et al*, 1997; Edmonson *et al*, 1998; Lane *et al*, 1995; Hirsch *et al*, 1997; Kimata and Overbaugh, 1997). Microglial passage of SIV in the generation of $SIV_{mac}182$ resulted in genetic changes in *env* which clustered in the V1 and V4 regions of gp120 (Lane *et al*, 1995). Furthermore, selection for or enrichment of these *env* quasispecies correlated with the presence of neuropathology, suggesting that *env* sequences may be important in contributing to neurovirulence. In a recent study by Kimata and Overbaugh (1997), a variant of SIVMne was generated in which cytopathicity mapped to the intracellular domain of the transmembrane (TM) portion of the *env* gene. These authors note that although truncation of the cytoplasmic tail of $SIV_{mac}239$ does not affect the cytopathicity of that virus, the presence of a full-length cytoplasmic tail was required for maximum SIVMne cytopathicity. In contrast, a recent study by Hirsch *et al* (1997) revealed that clones of SIVsmE543-3 isolated from brain but not peripheral blood had a predominance of *env* genes with stop codons in the TM domain. Their results indicate that *in vivo* selection occurs for truncated *env* sequences and suggests a possible functional role for prematurely truncated envelopes in brain but not blood. Both $SIV_{mac}251$ and $SIV_{mac}182$ clone 1100 encode a truncated form of gp41 (Naidu *et al*, 1988; Watry and Fox, in preparation). Clements and colleagues (Mankowski *et al*, 1997; Flaherty *et al*, 1997) have derived a neurovirulent clone, SIV/17E-Fr, which also has a truncation in gp41. Taken together, these results suggest that selection for neuroinvasive strains correlates with a truncated gp41. The significance of this observation is unclear, given that there have been reports that prematurely truncated gp41 occurs due to passage in human cells and reverts readily to full-length *in vivo* (Kodama *et al*, 1989; Hirsch *et al*, 1989). The question then arises of whether repair of the truncation codon in gp41 will result in a more neuroinvasive/neurovirulent virus. A study by Edmonson *et al* (1998) indicates that repair of the

stop codon in gp41 of $SIV_{mac}251$ results in a virus with slightly increased pathogenicity, in that survival time of macaques inoculated with the gp41-open virus had a mean survival time of 2.5 years, as compared to 7 years for the parental, gp41-truncated $SIV_{mac}251$. However, the question of neuroinvasiveness/neuropathogenicity was not addressed in this study. Clearly, this issue warrants further study. We have multiple clones of $SIV_{mac}182$ available (Watry and Fox, in preparation), and are currently investigating their growth properties in BMEC *in vitro*.

Here we have shown that growth in BMEC may predict neuroinvasiveness *in vivo*, and furthermore, that brain passage of virus results in the generation of neuroinvasive strains which demonstrate an increased efficiency of BMEC infection *in vitro*. Since growth in BMEC may predict neuroinvasiveness, we propose to map determinants of neuroinvasiveness through the generation of intertypic recombinants between strains which are not neuroinvasive (SIV_{mac} 239) and those which are weakly neuroinvasive ($SIV_{mac}251$) or strongly neuroinvasive ($SIV_{mac}182$) as measured by their abilities to infect and replicate in BMEC in our model system. Using these viral clones and others, we can also examine determinants of neuropathogenicity. A study by Flaherty *et al* (1997) implicates an arginine-to-glycine amino acid change at position 8854 of the TM portion of *env* (gp41) as being absolutely required for SIV replication in BMEC. With HIV infection of human BMEC *in vitro*, the determinants for endothelial cell growth mapped to a region of the genome which was distinct from those implicated in either T-cell line or macrophage tropism (Moses *et al*, 1996b). Rather, the differences in *env* sequences occuring in endothelial cell tropic versus non-tropic strains were consistent with the C1 region of gpl 20 being a major determinant of endothelial cell tropism. However, the cloned $SIV_{mac}251$ used in this study has a premature stop codon in gp41 at amino acid 8836 upstream of the putative endothelial-tropism residue, and therefore does not encode this residue in the gp41 protein, yet grows quite well in BMEC in culture. Thus, it is possible that a similar situation exists for SIV as for HIV, with additional determinants for endothelial cell tropism mapping to gpl20 and potentially other genes.

The ability of some neurovirulent SIV strains to enter endothelial cells mapped to *env* sequences in a study by Edinger *et al* (1997). In this study, the env proteins of SIV/17E-Fr was found to utilize the CCR5 chemokine receptor in a CD4-independent manner during infection of BMEC (which express both CCR5 and STRL33), in that infection was blocked by ligand to CCR5. Additionally, the env of SIV/17E-Fr was capable of mediating CD4-independent fusion and infection in indicator cell lines.

Interestingly, we observed that cloned $SIV_{mac}251$ failed to spread efficiently in BMEC cultures. $SIV_{mac}182$ clone 1100, in contrast, was either able to infect BMEC more efficiently, spread better, or both. This result indicates that growth in BMEC and ability to spread in BMEC are separable entities, and it will be interesting to see where these properties map. The capacity to be neurovirulent most likely maps in part to *env*. Flaherty *et al* (1997) found that macrophage tropism and neurovirulence were not necessarily correlated, in that a macrophage tropic clone was unable to infect BMEC or to cause brain pathology *in vivo*. Some of the differences between this virus and one which was able to infect BMEC and cause brain pathology mapped to the *env* gene. In addition, these authors found that an intact *nef* gene was important for productive infection of BMEC. *nef* has been well-characterized in terms of its ability to contribute to pathogenesis. $SIV_{mac}239$, with a premature stop codon in *nef*, reverts to an open gene through animal passage, indicating the importance of full-length *nef* for pathogenesis (Kestler *et al*, 1991). Flaherty *et al* (1997) found that viruses with truncated *nef* genes could not productively infect BMEC. Since *nef* may play a role in the establishment of latent HIV infections, it may also have a role in maintaining a latent virus reservoir (Binninger *et al*, 1991). This hypothesis, if true, may provide an explanation for the selection of full-length *nef* in those viruses which are able to infect BMEC. Thus, we would hypothesize that infection of BMEC *in vivo* results in a latent infection which provides a viral reservoir and contributes to mechanisms of AIDS dementia.

Using our *in vitro* model, we will attempt to generate an SIV_{mac} clone with neuroinvasive and neuropathogenic properties which will cause neurological manifestations in monkeys similar to those seen in AIDS dementia patients in a time frame suitable for study. BMEC are infectible *in vivo* and may represent a reservoir of virus, in that there appears to be a persistent level of viral replication with minimal pathology in BMEC cultures infected with neuroinvasive strains of SIV. Accumulations of subtle effects of virus on BMEC might result in the symptomology of AIDS dementia, which almost always occurs as a late manifestation of HIV disease. Thus, infection of BMEC *in vivo* may provide a route for viral entry into CNS tissues, contribute to the disease process, or remain a source of virus throughout the course of infection. Several models have been proposed to explain the direct and indirect effect of BMEC infection *in vivo* (Moses *et al*, 1996a; Moses and Nelson, 1995; Fox *et al*, 1997). In one model, HIV infection of BMEC may contribute to the disruption of an intact BBB. The consequences of a disrupted BBB include increased permeability of the CNS to toxic cellular metabolites or viral products, as well as increased viral trafficking into the brain. In another model, infection of BMEC

results in the expression of cytotoxic cytokines or adhesion molecules, which would result not only in cellular damage, but might also contribute to the recruitment of inflammatory cells or infected cells into the brain parenchyma. Since both HIV and SIV infect BMEC *in vivo*, and virus must pass through these cells in order to gain access to the CNS, it is imperative to investigate the role of BMEC infection on HIV-induced mechanisms of dementia. We will explore some of these hypotheses through the use of our *in vitro* model, in a continuing endeavor to understand some of the mechanisms responsible for AIDS dementia complex.

Materials and methods

Cells

The human T-B cell hybrid line 174 × CEM cells (NIH AIDS Research and Reference Reagent Program #272) were cultured in complete RPMI medium (RPMI supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). Macaque peripheral blood mononuclear cells (PBMC) were isolated from whole, heparinized blood of SIV-, simian foamy virus- and simian type D retrovirus-negative rhesus macaques (*M. mulatta*) by Histopaque (Sigma, St Louis, MO) centrifugation. PBMCs were cultured in complete RPMI medium supplemented to 20% heat-inactivated fetal bovine serum and with 3% human T-STIM (Collaborative Biomedical Products). 293T cells (Bartz *et al*, 1996) were cultured in complete DMEM (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). s-MAGI cells (Chackerian *et al*, 1995) were maintained under G418 (0.2 mg/ml) and hygromycin (50 U/ml) selection as described (Chackerian *et al*, 1995) in complete DMEM.

Virus stocks

5×10^5 293T cells in 6-well plates (Costar) were transfected with 2 μg of $SIV_{mac}251$ Phage DNA (molecular clone) (NIH AIDS Research and Reference Reagent Program #213) using a modified calcium chloride transfection protocol (Bartz *et al*, 1996). Cells were cultured in complete DMEM for 3 days, when supernatant virus was harvested, filtered through 0.22 μM pore size filters (Nalgene) and stored in 1 ml aliquots at −70°C. 293T transfection supernatants were amplified through either 174 × CEM cells or simian PBMCs stimulated with 5 μg/ml phytohemagglutinin (PHA; Sigma) for 2 days. Infection was monitored by supernatant p27 levels as measured by commercial ELISA (Coulter Corporation, Miami, FL). Amplified virus was harvested as above. An uncloned stock of $SIV_{mac}251$ and the molecular clone $SIV_{mac}239$ were obtained from Dr Ronald Desrosiers and propagated in 174 × CEM cells. The uncloned strain $SIV_{mac}182$ was isolated after serial animal passage of brain microglia as previously described (Watry *et al*, 1995). Details of the cloning and analysis of SIV proviral sequences from the brain of monkey 182 will be published elsewhere (Watry and Fox, in preparation). In brief, using nested PCR, genomic DNA from the brain of rhesus 182 was PCR amplified with primers specific for the 3′ half of SIV_{mac} strains, and the product cloned into pCR2.1 (Invitrogen, Carlsbad, CA). The *Eco*RV/*Sph*I fragment containing the insert was then subcloned into the *Eco*RV/*Sph*I sites of Litmus 38 (New England Biolabs, Beverly, MA). One of the resulting plasmids, denoted 1100, was subjected to sequence analysis and used for the experiments described here. The 1100 plasmid was linearized with *Sph*I and ligated to another *Sph*I-linearized plasmid (p239SpSp5′; AIDS Research and Reference Reagent Program) containing the 5′ half of $SIV_{mac}239$. The ligation product was transfected by the DEAE-dextran technique into 174 × CEM cells, and a 24 h cell-free supernatant collected at day 11 post-transfection. The tissue culture infectious dose (TCID) per ml of each virus preparation was determined by s-MAGI assay as previously described (Chakerian *et al*, 1995). In brief, s-MAGI cells were inoculated with serial viral dilutions in the presence of 10 μg/ml DEAE-dextran. The cells were stained for β-gal expression 3 days post-inoculation.

In situ *hybridization*

Rhesus macaque 182, free of type D simian retroviruses and herpes B virus was obtained from an isolated colony on Key Lois (Charles River Laboratories, Wilmington, MA) and housed in a containment facility at The Scripps Research Institute. All animal procedures were performed under ketamine anesthesia. The animal was inoculated with a stock of microglia-associated SIV (Watry *et al*, 1995; Lane *et al*, 1995) via injection of the saphenous vein. The animal became productively infected, as determined by plasma viral antigenemia and recovery of virus from PBMC. The animal was sacrificed by lethal anesthesia at day 69 post-inoculation due to rapidly progressive disease, after which it was perfused intracardially with phosphate-buffered saline. Pathological examination of the brain revealed typical findings of SIV encephalitis (Watry *et al*, 1995). All animal work was approved by The Scripps Research Institute's Animal Care committee. The Scripps Research Institute is accredited by the American Association for the Accreditation of Laboratory Animal Care. *In situ* hybridization was performed on formalin-fixed, paraffin-embedded tissue (Lane *et al*, 1996). 6 μm sections were prepared by Proteinase K digestion (50 μg/ml,

20 min at 37°C) and prehybridized under RNase-free conditions in 1.2 M NaCl, 20 mM Tris pH 7.5, 10 mM EDTA, 1 × Denhardt's, 1 mg/ml yeast RNA and 10 mM DTT, followed by hybridization with an ^{35}S-labeled single-strand RNA probe (containing 865 bp of the SIV *nef* gene) in prehybridization buffer made 40% in formamide and 10% dextran sulfate. Following overnight incubation at 46°C, slides were washed in 0.2 × SSC, 2 mM EDTA and 12 mM βME, RNase-treated (20 μg/ml, 30 min at room temperature), washed twice more at 50°C, dried and covered with Kodak NTB2 emulsion (diluted 1 : 1 with water), and stored in the dark at 4°C. Slides were developed after 5 to 7 days, counterstained with hematoxylin and eosin, coverslipped and examined. Antisense probes were used for the experimental sections; control sections were hybridized to sense riboprobes and to irrelevant riboprobes.

Isolation of simian brain microvascular endothelial cells

Simian brain microvascular endothelial cells (BMEC) were isolated from cortex of SIV-, simian foamy virus- and simian type D retrovirus-negative rhesus macaques (*M. mulatta*; macaque tissues generously provided by Michael Axthelm and Scott Wong, Oregon Regional Primate Research Center) as previously described for human tissue (Moses *et al*, 1993). In brief, simian brain tissue obtained at necropsy was rinsed and the meninges and all large blood vessels removed. Tissue was then cut into small pieces and digested with 50 mg/ml collagenase (Sigma) for 30 min at 37°C. During the collagenase digestion, tissue was manually dispersed by tituration through pipets of decreasing size. After digestion and dispersion, the tissue slurry was passed through a 70 μM cell strainer (Falcon) and clarified by centrifugation at 2500 r.p.m. for 20 min over a 25% BSA (Sigma) cushion. The pelleted capillaries were rinsed and adhered to a glass bead (1 mm size; Fisher Scientific) column for 30 min at 37°C. Both flow-through and adherent fractions were plated into Primaria (Falcon) plates in complete endothelial medium (Endothelial SFM (Gibco) supplemented with 10% human AB serum (Sigma), 25 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products), 40 μg/ml heparin (Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). After several days in culture, individual endothelial cell colonies were subcloned by trypsinization with cloning rings and passaged three times before cryopreservation. This step was included to preclude macrophage contamination. Endothelial cell purity was also assayed by positive immunoreactivity for von Willebrand factor VIII-related antigen (vWF) (DAKO, Carpenteria, CA) and endoglin (Antigenix America) and absence of immunoreactivity to smooth muscle actin (1A4; DAKO), TE-7 (Harlan Bioproducts, Indianapolis, IN) and GFAP (DAKO) antibodies. Secondary antibodies were goat anti-rabbit, rhodamine conjugate or goat anti-mouse, FITC conjugate (Biosource International, Camarillo, CA).

Viral inoculation of BMEC

Subconfluent simian BMEC at passage 5 or 6 were inoculated with between 1.5×10^4 to 7×10^4 TCID of SIV_{mac} in complete RPMI containing 2 μg/ml polybrene (Sigma). Mock-infected cells received complete RPMI + polybrene alone. Viral inoculum was removed after 4 h at 37°C by washing three times with HBSS and the medium replaced with complete endothelial medium. The supernatant was removed daily for determination of cell-free virus, the cultures were washed three times with HBSS and the medium was replaced. For viral growth curves in BMEC, plates were infected in triplicate and daily supernatants were assayed for the presence of p27 by commercial ELISA antigen capture assay (Coulter).

Immunofluorescence

Staining of BMEC for the presence of viral p27 or for endothelial cell markers was detected by indirect immunofluorescence. Cell monolayers were fixed at room temperature for 10 min in 95% ethanol/5% glacial acetic acid, permeabilized for 5 min at room temperature with 0.3% Triton X-100 in PBS and blocked for 20 min at 37°C with 20% normal goat serum in PBS. Cells were incubated with a 1 : 100 dilution of primary antibody (see below) for 1 h at 37°C and subsequently for 1 h at 37°C with a 1 : 100 dilution of either rhodamine-conjugated goat anti-rabbit or fluorescein-conjugated goat anti-mouse secondary antibody (Biosource International). Cells were examined for staining using a Nikon Optiphot inverted fluorescence microscope. Primary antibodies were as follows: BMEC culture purity was assessed using a mouse monoclonal to human von Willebrand factor VIII-related antigen (vWF) (DAKO) and by positive staining for endoglin (TGFβ receptor; Antigenix America). Further, cultures were assessed for the absence of immunoreactivity to smooth muscle actin (1A4; DAKO), TE-7 (Harlan Bioproducts, Indianapolis, IN) and GFAP (DAKO) antibodies. The presence of SIV was detected via immunoreactivity with a mouse monoclonal antibody to p27 (Advanced Biotechnologies, Columbia, MD).

Acknowledgements

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Pro-

gram, Division of AIDS, NIAID: 174 × CEM cells from Dr Peter Cresswell and p239SpSp5′ from Dr Ronald Desrosiers. This work was supported by grants from the NIH, MH47680 and MH55836 to HSF and RO1 MH55837 to JAN. We thank Rebecca Ruhl for technical assistance. We thank Michael Axthelm and Scott Wong for rhesus tissues.

References

Anderson MG, Hauer D, Sharma DP, Joag SV, Narayan O, Zink MC, Clements JE (1993). Analysis of envelope changes acquired by $SIV_{mac}239$ during neuroadaptation in rhesus macaques. *Virology* **195:** 616–626.

Bartz SR, Rogel ME, Emerman M (1996). Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2 accumulation by a mechanism which differs from DNA damage checkpoint control. *J Virol* **70:** 2324–2331.

Binninger D, Ennen J, Bonn D, Norley SG, Kurth R (1991). Mutational analysis of the simian immunodeficiency virus SIVmac *nef* Gene *J Virol* **65:** 5237–5243.

Budka H (1991). Neuropathology of human immunodeficiency virus infection. *Brain Pathol* **1:** 163–175.

Budka H, Wiley CA, Kleihues P *et al.* (1991). HIV-associated disease of the nervous system: Review of nomenclature and proposal for neuropathology-based terminology. *Brain Pathol* **1:** 143–152.

Chackerian B, Haigwood NL, Overbaugh J (1995). Characterization of a CD4-expressing macaque cell line that can detect virus after a single replication cycle and can be infected by diverse simian immunodeficiency virus isolates. *Virology* **213:** 386–394.

Chakrabarti L, Hurtrel M, Maire M-A, Vazeux R, Dormont D, Montagnier L, Hurtrel B (1991). Early viral replication in the brain of SIV-infected rhesus monkeys. *Am J Pathol* **139:** 1273-1280.

Daniel MD, Letvin NL, King NW, Kannagi M, Sehgai PK, Hunt RD, Kanki PJ, Essex M, Desrosiers RC (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228:** 1201–1204.

Daniel MD, Lewin NL, Sehgal PK, Hunsmann G, Schmidt DK, King NW, Desrosiers RC (1987). Long-term persistent infection of macaque monkeys with the simian immunodeficiency virus. *J Gen Virol* **68:** 3183–3189.

Desrosiers R (1990). The simian immunodeficiency viruses. *Ann Rev Immunol* **8:** 557.

Desrosiers RC, Hansen-Moosa A, Mori K, Bouvier DP, King NW, Daniel MID, Ringler DJ (1991). Macrophage tropic variants of SIV are associated with specific AIDS-related lesions but are not essential for the development of AIDS. *Am J Pathol* **139:** 29–35.

Edinger AL, Mankowski JL, Doranz BJ, Margulies BJ, Lee B, Rucker J, Sharron M, Hoffrnan TL, Berson JF, Zink MC, Hirsch VM, Clements JE, Doms RW (1997). CD4-independent, CCR5-dependent infection of brain capillary enclothelial cells by a neurovirulent simian immunodeficiency virus strain. *Proc Natl Acad Sci USA* **94:** 14742–14747.

Edmonson P, Murphey-Corb M, Martin LN, Delahunty C, Heeney J, Kornfeld H, Donahue PR, Learn GH, Hood L, Mullins JI (1998). Evolution of a simian immunodeficiency virus pathogen. *J Virol* **72:** 405–414.

Flaherty MT, Hauer DA, Mankowski JL, Zink MC, Clements JE (1997). Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. *J Virol* **71:** 5790–5798.

Fox HS, Gold LH, Henriksen SJ, Bloom FE (1997). Simian immunodeficiency virus: A model for neuroAIDS. *Neurobiol of Disease* **4:** 265–274.

Gabuzda DH, Ho DD, de la Monte SM, Hirsch MS, Rota TR, Sobel RA (1986). Immunohistochernical identification of HTLV III antigen in brains of patients with AIDS. *Ann Neurol* **20:** 289–295.

Glass JD, Wesselingh SL, Seines OA, McArthur JC (1993). Clinical-neuropathological correlation in HIV-associated dementia. *Neurology* **43:** 2230–2237.

Goldstein GW, Lorris Betz A (1983). Recent advances in understanding brain capillary function. *Neurol* **14:** 389–395.

Hirsch VM, Edmonson P, Murphey-Corb M, Arbielle B, Johnson PR, Mullins JI (1989). SIV adaptation to human cells. *Nature* **341:** 572–574 (Letter).

Hirsch V, Adger-Johnson D, Campbell B, Goldstein S, Brown C, Elkins WR, Montefiori DC (1997). A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543-3. *J Virol* **71:** 1608–1620.

Joo F 1985. The Blood-Brain Barrier *in vitro*: Ten years of research on microvessels isolated from the brain. *Neurochem Int* **7:** 1–25.

Kestler HW III, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC (1991). Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. *Cell* **65:** 651–662.

Kimata JT, Overbaugh J (1997). The cytopathicity of a simian immunodeficiency virus mne variant is determined by mutations in gag and env. *J Virol* **71:** 7629–7639.

Kodama T, Wooley DP, Naidu YM, Kestler HW, Daniel MD, Desrosiers RC (1989). Significance of premature stop codons in *env* of simian immunodeficiency virus. *J Virol* **63:** 4709–4714.

Koenig S, Gendleman HE, Orenstein JM, Del Canto MC, Pezeshkpour GM, Yungbiuth M, Jenotta F, Akasmit A, Martin MA, Fauci AS (1986). Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233:** 1089–1093.

Lackner A, Smith M, Munn R, Martfeld D, Gardner M, Marx P, Dandekar S (1991). Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *Am J Pathol* **139:** 609.

Lane TE, Buchmeier MJ, Watry DD, Jakubowski DB, Fox HS (1995). Serial passage of microglia SIV results in selection of homogeneous *env* quasispecies in the brain. *Virology* **212:** 458–465.

Lane TE, Buchmeier MJ, Watry DD, Fox HS (1996). Expression of inflammatory cytokines and inducible nitric oxide synthase in brains of SIV-infected rhesus monkeys: applications to HIV-induced disease. *Molecular Medicine* **2:** 27–37.

Lazarini F, Seilhean D, Rosenblum O, Suarez S, Conquy L, Uchihara T, Sazdovitch V, Mokhtari K, Maisonobe T, Bousin F, Katlarna C, Bricaire F, Duyckaerts C, Hauw J-J (1997). Human immunodeficiency virus type 1 DNA and RNA load in brains of demented and nondemented patients with acquired immunodeficiency syndrome. *J Neurovirol* **3:** 299–303.

Letvin NL, Daniel MD, Sehgal PK, Desrosiers RC, Hunt RD, Waldron LM, MacKey JJ, Schmidt DK, Chalifoux LV, King NW (1985). Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STL-V-III. *Science* **230:** 71–73.

Mankowski JL, Flaherty MT, Spelman JP, Hauer DA, Didier PJ, Martin Amedee A, Murphey-Corb M, Kirstein LM, Munoz A, Clements JE, Zink MC (1997). Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. *J Virol* **71:** 6055–6060.

Mankowski JL, Spelman JP, HG Ressetar HG, Strandberg JD, Laterra J, Carter DL, Clements JE, Zink MC (1994). Neurovirulent simian immunodeficiency virus replicates productively in endothelial cells of the central nervous system in vivo and in vitro. *J Virol* **68:** 8202–8208.

McArthur JC, Hoover DR *et al*. (1993). Dementia in AIDS patients: incidence and risk factors. *Neurology* **43:** 2245–2252.

Moses AV, Nelson JA (1994). HIV Infection of human brain capillary endothelial cells–implications for AIDS dementia. *Adv in Neuroimmunol.* **4:** 239–247.

Moses AV, Nelson JA (1995). An organ model for HIV infection of the blood-brain barrier. In *Technical Advances in AIDS research in the human nervous system.* Plenum Press, New York pp. 205–221.

Moses AV, Bloom FE, Pauza CD, Nelson JA (1993). Human immunodeficiency virus infection of human brain capillary endothelial cells occurs via a CD4/galactosylceramide-independent mechanism. *Proc Natl Acad Sci* **90:** 10474–10478.

Moses AV, Stenglein S, Nelson JA (1996a). HIV infection of the brain microvasculature and its contribution to the AIDS dementia complex. *J NeuroAIDS* **1:** 85–96.

Moses AV, Stenglein SG, Strussenberg JG, Wehrly K, Chesebro B, Nelson JA (1996b). Sequences regulating tropism of human immunodeficiency virus type 1 for brain capillary endothelial cells map to a unique region on the viral genome. *J Virol* **70:** 3401–3406.

Moses AV, Williams S, Heneveld ML, Strussenberg J, Rarick M, Loveless M, Bagby G, Nelson JA (1996c). HIV infection of bone marrow endothelium reduces induction of stromal hematopoetic growth factors. *Blood* **87:** 919–925.

Murray EA, Rausch DM, Lendvay J, Sharer LR, Eiden LE (1992). Cognitive and motor impairment associated with SIV infection in rhesus monkeys. *Science* **255:** 1246–1249.

Naidu YM, Kestler HW III, Li Y, Butler CV, Silva DP, Schmidt DK, Troup CD, Sehgal PK, Sonigo P, Daniel MD, Desrosiers RC (1988). Characterization of infectious molecular clones of simian immunodeficiency virus (SIV_{mac}) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV_{mac}. *J Virol* **62:** 4691–4696.

Navia BA, Jordan BD, Price RW (1986a). The AIDS dementia complex: I. Clinical features. *Ann Neurol* **19:** 517–524.

Navia BA, Cho E-S, Petito CK, Price RW (1986b). The AIDS dementia complex: II. Neuropathology. *Ann Neurol* **19:** 525–535.

Petito CK, Cash KS (1992). Blood-brain barrier abnormalities in the acquired immunodeficiency syndrome: immunohistochemical localization of serum proteins in postmortem brain. *Ann Neurol* **32:** 658–666.

Power C, Kong P-A, Crawford TO, Wesselingh S, Glass JD, McArthur JC, Trapp BD (1993). Cerebral white matter changes in acquired immunodeficiency syndrome dementia: alterations of the blood-brain barrier. *Ann Neurol* **34:** 339–350.

Prospero-Garcia, O Gold LH, Fox HS, Polis I, Koob GF, Bloom FE, Henriksen SJ (1996). Microglia-passaged simian immunodeficiency virus induces neurophysiological abnormalities in monkeys. *Proc Natl Acad Sci USA* **93:** 14158–14163.

Rhodes R (1991). Evidence of serum-protein leakage across the blood-brain barrier in the acquired immunodeficiency syndrome. *J Neuropathol Exp Neurol* **50:** 171–183.

Saito Y, Sharer LR *et al*. (1994). Overexpression of nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tissues. *Neurology* **44:** 474–481.

Sharer LR (1992). Pathology of HIV-1 infection of the central nervous system. A review. *J Neuropathol Exp Neurol* **51:** 3–11.

Sharer L, Michaels J, Murphey-Corb M, Hu F-S, Kuebler D, Martin L, Baskin G (1991). Serial Ppathogenesis study of SIV brain infection. *J Med Primatol* **20:** 211.

Sharma DP, Anderson M, Zink MC, Adams RJ, Donnenberg AD, Clements JE, Narayan O (1992a). Pathogenesis of acute infection in rhesus macaques with a lymphocyte-tropic strain of simian immunodeficiency virus. *J Inf Dis* **166:** 738–746.

Sharma DP, Zink MC, Anderson M, Adams R, Clements JE, Joag SV, Narayan O (1992b). Derivation of neurotropic simian immunodeficiency virus from exclusively lymphocytetropic parental virus: pathogenesis of infection in macaques. *J Virol* **66:** 3550–3556.

Simon M, Chalifoux L, Ringler D (1992). Pathologic features of SIV-induced disease and the association of macrophage infection with disease evolution. *AIDS Res Hum Retroviruses* **8:** 327.

Singer EJ, Syndulko K, Fahy-Chandon B, Schmid P, Conrad A, Tourellotte WW (1994). Intrathecal IgG synthesis and albumin leakage are increased in subjects with HIV-1 neurological disease. *J AIDS* **7:** 265–271.

Smith M, Sutjipto S, Lackner A (1994). Intrathecal synthesis of IgG in simian immunodeficiency virus. *AIDS Res Hum Retroviruses* **10:** 81–89.

Spencer DC, Price RW (1992). Human immunodeficiency virus and the central nervous system. *Annu Rev Microbiol* **46:** 655–693.

Stephens EB, Lui ZQ, Zhu GW, Adany I, Joag SV, Foresman L, Berman NJ, Narayan, O (1995). Lymphocyte-tropic simian immunodeficiency virus causes persistent infection in the brains of rhesus monkeys. *Virology* **213:** 600–614.

Takahashi K, Wesselingh SL, Griffin DE, McArthur JC, Johnson RT, Glass JD (1996). Localization of HIV-1 in human brain using polymerase chain reaction/in situ hybridization and immunocytochemistry. *Ann Neurol* **39:** 705–711.

Tornatore C, Chandra R, Berger JR, Major EO. (1994). HIV-1 infection of subcortical astrocytes in the paediatric AIDS. *Neurology* **44:** 481–487.

Turner R, Beckstead JH, Warnke RA, Wood GS (1987). Endothelial cells' phenotypic diversity. In situ demonstration of immunologic and enzymatic heterogeneity that correlates with specific morphologic Subtypes. *Am J Clin Pathol* **87:** 569–575.

Vazeux R, Brousse N, Jarry A *et al*. (1987). AIDS subacute encephalitis: identification of HIV-infected cells. *Am J Pathol* **126:** 403–410.

von Herrath M, Oldstone MBA, Fox HS (1995). Simian immunodeficiency virus (SIV)-specific CTL in cerebrospinal fluid and brains of SIV-infected rhesus macaques. *J Immunol* **154:** 5582–5589.

Ward JM, O'Leary TJ, Baskin GB, Beveniste R, Harris CA, Nora PL, Rhodes RH (1987). Immunohistochemical localization of human and immunodeficiency viral antigens in fixed tissue sections. *Am J Pathol* **127:** 199–205.

Watry D, Lane TE, Streb M, Fox HS (1995). Transfer of neuropathogenic simian immunodeficiency virus with naturally infected microglia. *Am J Pathol* **146:** 914–923.

Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MBA (1986). Cellular localization of human immunodeficiency virus infection within the brains of acquired immunodeficiency syndrome patients. *Proc Natl Acad Sci USA* **83:** 7089–7093.

Zhu GW, Lui ZQ, Joag SV, Pinson DM, Adany I, Narayan O, McClure HM, Stephens EB (1995). Pathogenesis of lymphocyte-tropic and macrophage-tropic SIV_{mac} infection in the brain. *J Neurovirol* **1:** 78–91.