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Neuroinvasion by simian immunodeficiency virus coincides with increased numbers of perivascular macrophages/microglia and intrathecal immune activation

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During peak viremia and initial antibody response, rhesus macaques infected with pathogenic and nonpathogenic isolates of SIV show distinct differences in viral load and tissue distribution. Animals infected with pathogenic isolates of SIV invariably have virus in the CSF and brain parenchyma by two weeks postinoculation, whereas animals infected with nonpathogenic isolates do not. Mechanisms underlying neuroinvasion by SIV and HIV are unknown, but recruitment of latently infected mononuclear cells from the peripheral circulation (Trojan horse theory) is frequently proposed. Circulating monocytes, from which perivascular macrophage/microglia are derived, are a likely vehicle for cell-associated transport of virus across the blood-brain barrier. This transport and the kinetics of perivascular macrophage/microglial turnover in the CNS likely depend on endothelial and leukocyte adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), which has previously been shown to be upregulated on cerebrovascular endothelium in SIV encephalitis. To investigate the role of peripheral monocyte recruitment into the perivascular macrophage/microglial cell pool at the time of initial viral neuroinvasion, we examined the temporal relationships among perivascular macrophage/microglia density, endothelial VCAM-1 expression and localization of viral nucleic acid in the CNS of macaques acutely infected with pathogenic and nonpathogenic molecular clones of SIV. The concentration of CSF quinolinic acid, a marker of intrathecal immune and macrophage activation, was examined concurrently. We found that significant increases in the density of perivascular macrophages/microglia coincided with viral neuroinvasion and marked elevations in CSF quinolinic acid. Furthermore, combined *in situ* hybridization and immunohistochemistry demonstrated that infected perivascular cells were macrophages/microglia. These findings provide evidence suggesting that neuroinvasion occurs through an influx of infected monocytes which take up residence in the CNS as perivascular macrophages/microglia. VCAM-1 expression, however, was not clearly correlated with these events, thus its contribution to initial viral neuroinvasion is unclear.

Keywords: neuroAIDS; leukocyte traffick; quinolinic acid; image analysis

Introduction

Encephalitis is a common debilitating consequence of HIV infection. Studies have shown that, in the acute stage of disease, virus is frequently present in the cerebrospinal fluid (CSF) of humans infected with HIV (Chiodi *et al*, 1988; Ho *et al*, 1985). Similarly, in the SIV/macaque model of AIDS, virus has been demonstrated in the CSF and brain parenchyma within 2 weeks of infection (Chakrabarti *et al*, 1991; Lackner *et al*, 1994). Despite the

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speed and frequency with which HIV and SIV enter the central nervous system (CNS) of their respective hosts, the mechanisms underlying viral entry into the CNS are unknown. The Trojan horse theory developed largely from work with Visna virus in sheep is a commonly proposed mechanism (Peluso *et al*, 1985). According to this theory, peripheral blood mononuclear cells, a portion of which are latently infected, carry virus across an intact blood–brain–barrier (BBB) and take up residence in the perivascular space or brain parenchyma where they actively produce virus. Support for this theory includes studies involving HIV in humans and SIV in rhesus macaques which demonstrate that: (1) parenchymal microglia and perivascular macrophages/microglia are the only resident cells of the CNS that support productive HIV/SIV infection (Brinkmann *et al*, 1992; 1993; Koenig *et al*, 1986; Lackner *et al*, 1991; Vazeux *et al*, 1987); (2) macrophage-competent variants are associated with development of encephalitis (Desrosiers *et al*, 1991; Vazeux *et al*, 1987); (3) neuroinvasion by SIV is not associated with a gross disruption of the BBB (Smith *et al*, 1995); (4) the distribution of SIV-infected cells early in infection is almost exclusively perivascular (Smith *et al*, 1995); and (5) direct intracerebral inoculation of SIV in rhesus monkeys fails to alter the distribution of virus and may delay the course of CNS disease compared to i.v. inoculation (Boche *et al*, 1995; Chakrabarti *et al*, 1991). If the Trojan horse mechanism is the primary means through which SIV and HIV enter the CNS, elucidating which stimuli are necessary for transmigration of monocytes across the BBB is of critical importance in understanding the neuropathogenesis of AIDS.

Microglia and macrophages are derived from a common bone marrow progenitor cell and both are capable of antigen presentation (Hickey and Kimura, 1988). Studies in rodents indicate that most parenchymal microglia arrive in the CNS during gestation, express few of the antigens associated with monocyte/macrophages and persist with very low turnover and limited capacity for cell division (Lawson *et al*, 1992). In contrast, perivascular macrophages/microglia have a more activated phenotype and express a larger repertoire of antigens common to macrophages elsewhere in the body. In addition, they turn over more frequently and are replaced by circulating monocytes throughout life (Perry, 1994). Furthermore, studies in the Lewis rat have demonstrated that recruitment of perivascular macrophages/microglia can be accelerated during CNS inflammation, while parenchymal microglia are only rarely replaced by hematogenous cells (Lassman *et al*, 1993).

The CNS responds to inflammatory stimuli in unique ways, rendering extrapolation from other tissues inappropriate (Andersson *et al*, 1992a). Consequently, we may expect different patterns of chemoattractant and adhesion molecule expression in CNS inflammation when compared to inflammation involving other tissues. We have shown that endothelial vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule belonging to the immunoglobulin superfamily which is induced by proinflammatory cytokines, is upregulated in the CNS of macaques with SIV encephalitis (Sasseville *et al*, 1992). Similarly, VCAM-1 expression parallels upregulation of proinflammatory cytokines in brain tissue of HIV-1 infected individuals (Nottet *et al*, 1996). Additionally, we have shown that VCAM-1/$\alpha 4\beta 1$ integrin interactions mediate selective adhesion of monocytes to encephalitic brain sections *in vitro* (Sasseville *et al*, 1994).

Several studies indicate that HIV and SIV do not productively infect neurons, focusing much attention on indirect mediators of neuronal injury including quinolinic acid (Genis *et al*, 1992; Heyes *et al*, 1992; Ljubisa *et al*, 1994). In the CNS, activated macrophages/microglia are most likely the predominant source of quinolinic acid (QUIN). QUIN is a metabolite of L-tryptophan which has been implicated in the neuropathogenesis of HIV infection via its action at neuronal N-methyl D-aspartate receptors (Heyes *et al*, 1991). Thus CSF QUIN is a marker of intrathecal immune activation which also may have a functional role in the neuropathogenesis of SIV infection.

To investigate the role of peripheral blood monocyte recruitment to the CNS in SIV neuroinvasion, we examined the temporal relationships among perivascular macrophage/microglial density, cerebral endothelial VCAM-1 expression, CSF QUIN production, and localization of viral nucleic acid in the CNS of acutely SIV-infected rhesus macaques. To examine the impact of viral determinants on these parameters, groups of animals were inoculated with either pathogenic (SIV_{mac239}) or nonpathogenic ($SIV_{mac1A11}$) molecular clones of SIV and compared to macaques inoculated with SIV_{mac}, an uncloned biological isolate which consists of numerous quasispecies. The two pathogenic isolates (SIV_{mac} and SIV_{mac239}) cause a fatal AIDS-like disease including SIV encephalitis. In contrast, $SIV_{mac1A11}$ is nonpathogenic and does not induce encephalitis (Lackner *et al*, 1994; Smith *et al*, 1995).

We found that the numbers of perivascular macrophages/microglia were highest at two weeks postinoculation in animals infected with the pathogenic isolates of SIV, concomitant with viral neuroinvasion and peak CSF QUIN levels. The nonpathogenic clone induced no significant changes in QUIN production or density of perivascular macrophages/microglia when compared to uninfected control animals and failed to invade the CNS. These data suggest that recruitment of peripheral blood monocytes to the perivascular

macrophage/microglial pool during the first few weeks of infection is a likely mechanism through which pathogenic SIV enters the CNS.

Results

Histopathology

Results of histopathologic examination of the CNS are summarized in Table 1. All animals with one exception (24891) were sacrificed before clinical signs of disease developed. However, histologic changes were observed in the CNS of several animals infected with SIVmac or SIVmac239. Lesions consisted of a mild meningoencephalitis with multifocal, perivascular and meningeal mononuclear cell infiltrates and rare glial nodules. While randomly distributed throughout the CNS, inflammatory cell infiltrates were more commonly observed in white matter than cortical or deep grey matter as previously described (Chakrabarti *et al*, 1991; Lackner *et al*, 1991; Smith *et al*, 1995). These changes were found most consistently in animals sacrificed at 2 weeks postinoculation (4/4) and diminished in frequency thereafter (1/4 at 8 weeks, 2/4 at 13 weeks and 1/4 at 23 weeks). Animal 24891 was sacrificed when moribund at 22 weeks postinoculation following rapid disease progression. This was the only animal to develop SIV encephalitis with characteristic parenchymal multinucleate giant cells. No lesions were observed in CNS tissues of animals inoculated with $SIV_{mac1A11}$.

Viral isolation

Results of viral isolation from PBMC and CSF from these animals have been reported in detail previously (Lackner *et al*, 1994). Briefly, animals inoculated with pathogenic virus were persistently viremic by 1 week except for one animal inoculated with SIV_{mac239} (24231). By 2 weeks postinoculation all animals inoculated with pathogenic virus were viremic and remained so throughout the study. Macaques inoculated with $SIV_{mac1A11}$ were viremic by 2 weeks postinoculation but the viremia was transient and no virus was isolated from these animals after four weeks of infection.

In the CSF, virus was isolated from all animals inoculated with SIV_{mac} or SIV_{mac239} by 2 weeks postinoculation. Between 8 and 13 weeks postinoculation, virus was isolated from only five of 27 CSF cultures collected from animals inoculated with pathogenic virus. By 21 weeks postinoculation, CSF cultures were again uniformly positive in all animals remaining in the study (n=4). In contrast, virus was never isolated from the CSF of animals inoculated with nonpathogenic $SIV_{mac1A11}$.

SIV-positive cells are perivascular in early infection

Paraffin sections of brain and spinal cord were examined for viral nucleic acids by *in situ* hybridization. Cells positive for viral nucleic acid were found almost exclusively in a perivascular location. No predilection for a specific anatomic region within the CNS was observed, although positive cells were more frequently observed in white matter compared to grey matter. Distribution of virus detected by *in situ* hybridization in CNS tissues of these animals has been previously reported (Smith *et al*, 1995). All four animals sacrificed 2 weeks after inoculation with SIV_{mac} and SIV_{mac239} had virus-positive cells closely associated with blood vessels (Figure 1a). In most instances, the number of perivascular macrophages/microglia positive for SIV by *in situ* hybridization was greatly exceeded by those which were uninfected (<10%, data not shown). Com-

Table 1 Neuropathologic findings

Virus	*Weeks postinoculation*	*Animal#*	*Major pathologic findings*
SIVmac	2	24318	Meningitis, mild; glial nodules, spinal cord
		24354	Meningoencephalitis with perivascular cuffs and glial nodules
	8	24260	NSL
		24634	NSL
	13	24373	NSL
		24377	Meningitis and mild choroid plexitis
	23	24891*	SIV encephalitis
		25003	NSL
SIVmac-239	2	23894	Meningitis, mild; perivascular cuffs
		23930	Meningitis, choroid plexitis; perivascular cuffs; mineralization, basal ganglia
	8	24231	Meningitis, choroid plexitis and perivascular cuffs
		24255	NSL
	13	24289	Meningomyelitis; perivascular cuffs; glial nodules, spinal cord
		24219	NSL
	23	24242	NSL
		24263	NSL

*Animal 24891 was euthanatized at 22 weeks pi with terminal simian AIDS. Animals inoculated with SIVmac1A11 had no significant pathologic findings. NSL=no significant lesions identified.

bined *in situ* hybridization/immunohistochemistry showed that infected cells were HAM56-positive, consistent with monocyte/macrophage derivation (Figure 1b). At later time points when remaining animals had developed SIV-specific immune responses (Smith *et al*, 1995), virus-positive cells were less frequent. The exception to this pattern was observed in animal 24891. This macaque had no detectable humoral immune response, became rapidly moribund with AIDS, and histologically had SIV encephalitis with characteristic multinucleate giant cells.

Perivascular macrophage/microglia density

To examine numbers of perivascular macrophage/microglia we performed immunohistochemistry using HAM56 (Figure 1c,d). For each vascular profile examined, the number of HAM56+ perivascular macrophages/microglia per millimeter of vessel length was determined by morphometric analysis (Figures 2 and 3). Animals infected with uncloned SIV_{mac} had significantly more perivascular macrophages than animals inoculated with SIV_{mac239} and $SIV_{mac1A11}$ or uninfected controls (P=0.006, F value=5.36). At 2 and 8 weeks postinoculation, numbers of perivascular macrophage/microglia in animals inoculated with SIV_{mac239} were greater than in animals inoculated with $SIV_{mac1A11}$ or uninfected controls, but these did not reach levels of statistical significance. In the pooled group of 28 animals, a statistically significant decline in the density of perivascular macrophages/microglia was observed by 13 weeks postinoculation (P=0.045, F value=2.90). Of all two-way comparisons among time intervals, differences between 0 and 2 weeks (P=0.008), 0 and 8 weeks (P=0.048), 2 and 13 weeks (P=0.046), and 2 and 23 weeks (P=0.023) reached levels of statistical significance.

Correlation of VCAM-1 expression and perivascular macrophage/microglial density

To examine the relationship between VCAM-1 expression and perivascular macrophage/microglial density in the acute phase of disease, representative frozen sections of cerebrum from each animal were scored for VCAM-1 expression as described (Silber *et al*, 1994) and correlated with perivascular macrophage/microglial density deter-

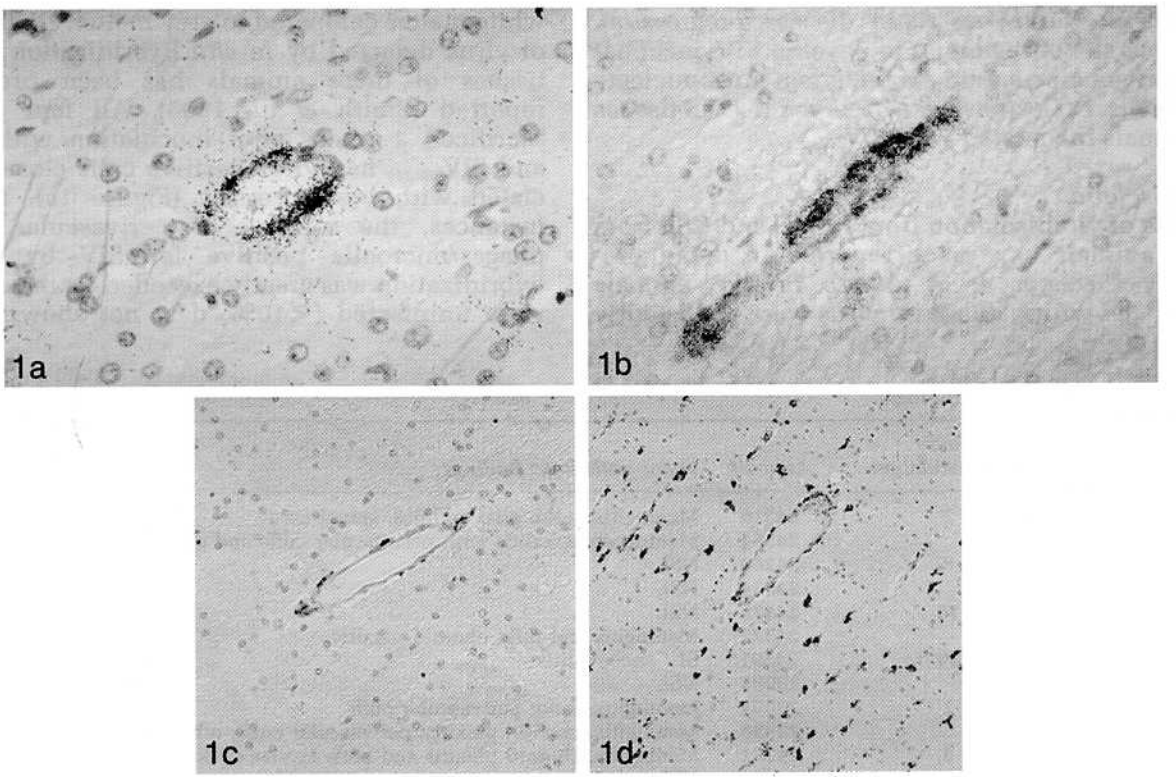

Figure 1 At 2 weeks postinoculation SIV-infected cells, shown by *in situ* hybridization, are consistently observed in intimate association with cerebral vasculature 210× (**a**). Combined *in situ* hybridization and immunohistochemistry (**b**) demonstrates that most SIV-infected cells associated with vessels express the monocyte/macrophage-specific antigen detected by HAM56 (red precipitate) 160×. In acutely infected animals (14 days postinoculation) HAM56 staining is confined to perivascular cells 110× (**c**). In contrast, animals with SIV encephalitis (24891) have, in addition to immunopositive perivascular macrophages/microglia, numerous HAM56-positive cells (activated parenchymal microglia and infiltrating macrophages) within cortical white matter 75× (**d**).

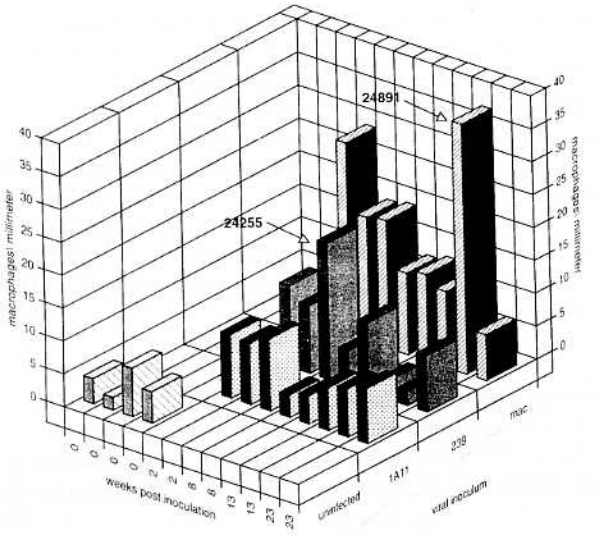

Figure 2 Perivascular macrophage density determined by image analysis of HAM56-immunostained sections of cortical white matter. Each column represents values for one animal, either uninfected (0 weeks) or inoculated with pathogenic uncloned SIV_{mac}, pathogenic cloned SIV_{mac239}, or nonpathogenic cloned $SIV_{mac1A11}$. Animals were serially sacrificed at 2, 8, 13 and 23 weeks postinoculation. Perivascular macrophage numbers were highest at 2 weeks postinoculation and declined significantly between 2 and 13 weeks. The animal that had the highest density of perivascular macrophages, 24891, was sacrificed at 22 weeks with severe SIV encephalitis. 24891 and 24255 (arrows), in which perivascular macrophage density was markedly increased compared to animals of similar inoculum and duration of infection, were the only infected animals that failed to mount detectable SIV-specific IgG responses.

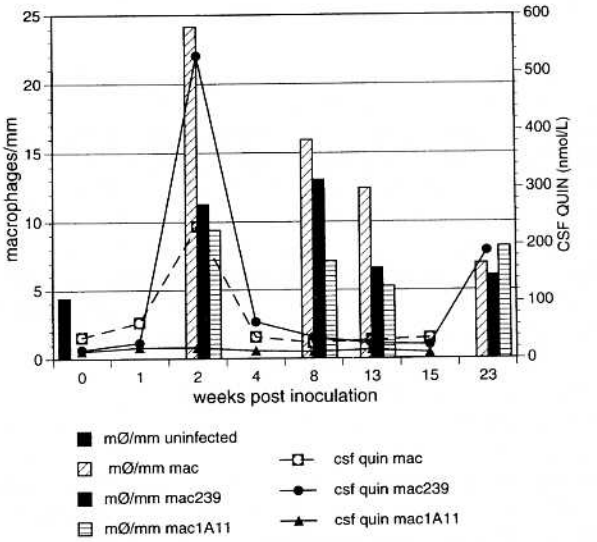

Figure 3 Temporal relationship between perivascular macrophage density and CSF QUIN concentration. Each column represents the average perivascular cell density of two animals sacrificed at each time point. Average perivascular macrophage density from four uninfected animals is shown at 0 weeks. CSF QUIN values represent averages of animals remaining alive at the indicated time point. Highest numbers of perivascular macrophages coincide with peak QUIN levels at 2 weeks postinoculation. Note the failure of SIVmac1A11 to induce changes in CSF QUIN levels. Excluded from the figure are values from animal 24891, which had terminal SIV encephalitis and markedly elevated values (QUIN level=901.7 nM/L, Perivascular macrophage density=38.01 cells/mm).

mined by image analysis. No regional/anatomic variations in the intensity of VCAM-1 immunostaining were observed. Modest, diffuse elevations in VCAM-1 expression were seen in animals inoculated with SIV_{mac239}, but changes in VCAM-1 expression in animals inoculated with SIV_{mac} and $SIV_{mac1A11}$ were inconsistent. Elevation in VCAM-1 expression was seen by 2 weeks postinoculation in animals inoculated with SIV_{mac239}, concurrent with the highest density of perivascular macrophages/microglia observed in the pooled group of SIV-inoculated animals. However, the level of VCAM-1 expression did not closely correlate with the numbers of perivascular macrophages/microglia in most animals. Two exceptions were animals 24891 and 24255, who had markedly increased VCAM-1 expression (4+) and increased density of perivascular macrophages/microglia (see Figure 2). Both animals were 'rapid progressors' and failed to mount a humoral immune response. Animal 24255 was killed at 8 weeks postinoculation prior to the onset of clinical signs, while 24891 developed terminal AIDS and SIV encephalitis. These findings support the previous observation that marked upregulation of VCAM-1 expression is associated with the development of SIV encephalitis.

Quinolinic acid levels correlate with perivascular macrophage/microglial density

CSF quinolinic acid, which is produced primarily by activated macrophages/microglia, was measured as a marker of intrathecal immune activation at 0, 1, 2, 4, 8, 13 and 23 weeks postinoculation. In animals inoculated with pathogenic viral isolates, QUIN values rose sharply (up to 1000-fold) by 2 weeks postinoculation coincident with viral neuroinvasion and an elevation in perivascular macrophage/microglial density (Figure 3). QUIN then rapidly decreased by 8 weeks postinoculation with the onset of an immune response (Smith *et al*, 1995). CSF QUIN in the $SIV_{mac1A11}$ group never increased significantly over preinoculation levels. Animal 24891, the only animal to develop SIV encephalitis during the 23-week study, showed delayed but persistent elevation of QUIN at 13 weeks postinoculation until sacrifice at 22 weeks. CSF QUIN was significantly greater in animals inoculated with either SIV_{mac} or SIV_{mac239} than in those inoculated with $SIV_{mac1A11}$. CSF QUIN was also significantly elevated in animals sacrificed at 2 weeks postinoculation compared with those sacrificed at 8-13 week time points. Detailed results and discussion of QUIN data from these animals have been previously reported (Smith *et al*, 1995).

Discussion

This study demonstrates that at 2 weeks postinfection, neuroinvasion by SIV coincides with significant increases in the density of perivascular macrophages/microglia in cerebral white matter. At this same time point, *in situ* hybridization experiments indicate that most virus-infected cells in the CNS are within perivascular spaces, intimately associated with adventitia of small vessels, a location most compatible with perivascular macrophages/microglia. Furthermore, combined *in situ* hybridization/immunohistochemistry shows that these virus-infected cells express the monocyte/macrophage-specific antigen recognized by HAM56. In addition, the widespread distribution of virus in the CNS, demonstrated by *in situ* hybridization, supports hematogenous dissemination. These data provide compelling evidence, as suggested by the Trojan horse theory (Peluso *et al*, 1985), that virus enters the CNS via recruitment of infected mononuclear cells from the systemic circulation.

In addition to increased density of perivascular macrophages/microglia at the time of neuroinvasion (2 weeks postinoculation), we observed a peak in CSF QUIN, indicative of intrathecal immune activation. Since activated macrophages/microglia are a potential source of QUIN in the CNS, the increased density of perivascular macrophages/microglia observed morphometrically likely contributes to this rise in QUIN. Increases in the density of perivascular macrophages/microglia, the presence of virus in the CNS, and elevations in CSF QUIN were limited to animals inoculated with SIV_{mac} and SIV_{mac239}. No virus or inflammatory lesions were found in the CNS of $SIV_{mac1A11}$-inoculated animals. Furthermore, in animals infected with $SIV_{mac1A11}$, the numbers of perivascular macrophages and CSF QUIN did not differ significantly from uninfected controls. Thus, $SIV_{mac1A11}$ is not neuroinvasive, despite sharing greater than 98% nucleotide homology with SIV_{mac239}. Whether failure of $SIV_{mac1A11}$ to invade the CNS is a function of lower viral loads, increased immunogenicity, or other factors is unclear. Unfortunately, it is difficult to attribute biologic behaviour of this virus to specific sequence variation since nucleotide differences are scattered throughout the genome. Analysis of such determinants necessitates utilization of molecularly cloned viruses with discrete genetic differences.

In animals inoculated with either SIV_{mac} or SIV_{mac239}, the density of perivascular macrophages/microglia decreased significantly by 13 weeks postinoculation. This decline was accompanied by less frequent isolation of virus from the CSF and a decrease in numbers of virus-positive cells detected by *in situ* hybridization (Lackner *et al*, 1994; Smith *et al*, 1995). Such findings are likely due to the modulating effect of the immune system on viral load in the CNS (Lackner *et al*, 1994; Smith *et al*, 1995; VonHerrath *et al*, 1995). In these animals, decline in viral load in the CNS paralleled a progressive decline in viral burden observed in lymph node, thymus, spleen, and peripheral blood (Lackner *et al*, 1994). One SIV_{mac239}-inoculated animal (24255) sacrificed at 8 weeks and one SIV_{mac}-inoculated animal (24891) sacrificed at 22 weeks, however, showed markedly elevated perivascular macrophage numbers compared to other animals of shared viral inoculum and duration of infection. Neither of these animals had detectable humoral immune responses and were 'rapid progressors' as previously described (Lackner *et al*, 1994; Reimann *et al*, 1994; Zhang *et al*, 1988).

These data indicate that there is a close relationship between increased density of perivascular macrophages/microglia, viral neuroinvasion, and intrathecal immune activation. This correlation suggests that infected cells are recruited to the CNS from the peripheral circulation. The involvement of specific chemoattractants and leukocyte-endothelial adhesion molecule interactions have not yet been elucidated. Our earlier observations, which show a clear correlation between endothelial expression of VCAM-1 and SIV encephalitis and a functional role for VCAM-1 in monocyte adhesion to encephalitic brain, suggest that VCAM-1 is a major contributor to leukocyte recruitment and the development of SIV encephalitis (Sasseville *et al*, 1992; 1994). Furthermore, expression of other adhesion molecules, including ICAM-1, P-selectin and E-selectin, was not found to correlate with SIV encephalitis or neuroinvasion early in infection (Sasseville *et al*, 1992; 1995). In the acute phase of disease, however, intensity of VCAM-1 immunostaining failed to show a close relationship with perivascular macrophage numbers, indicating that other factors may contribute to changes in the kinetics of perivascular macrophage recruitment. Marked increase in both VCAM-1 expression and perivascular macrophage/microglial density was observed in animals 24891 and 24255, both 'rapid progressors' with no detectable immune response. Animal 24891 was moribund at 22 weeks postinoculation with SIV encephalitis. Animal 24255 may have developed encephalitis had the disease process been permitted to progress. Increased VCAM-1 expression in animal 24891 supports previous work demonstrating the involvement of this adhesion molecule in the development of SIV encephalitis, but provides little additional insight into leukocyte recruitment mechanism(s) and viral neuroinvasion early in infection. It is likely that recruitment of leukocytes into the CNS and associated viral neuroinvasion are part of a complex process during which upregulation of VCAM-1 expression is only a single component. The role of chemokines (Furie and Randolph, 1995) should be examined. Recently, elevations in chemokine MIP-

1α and MIP-1β mRNA have been observed in the brains of HIV-infected patients with the AIDS dementia complex (Schmidtmayerova *et al*, 1995). These chemokines are potent attractants for monocytes and lymphocytes (Koch *et al*, 1994; Taub *et al*, 1993) and likely contribute to monocyte recruitment into the CNS of HIV-infected humans and SIV-infected macaques in conjunction with cytokine-induced adhesion molecules such as VCAM-1 (Nottet *et al*, 1996).

The data presented do not preclude involvement of infected lymphocytes in viral entry. In fact, work by Hickey *et al* (1991) has shown that activated lymphocytes (lymphoblasts) rapidly enter the CNS. However, lymphocytes are a minor component of the cellular infiltrates that characterize encephalitis caused by SIV or HIV. In addition, virtually all the lymphocytes present in the CNS lesions are $CD8^+$, $CD4^-$ (Lackner *et al*, 1991; Pumarola-Sune *et al*, 1987; Watry *et al*, 1995). Of more relevance to early events in SIV infection is recent work by vonHerrath *et al* (1995). This work has demonstrated that, while lymphocytes are present in the CNS as early as 1 week after infection, they are predominantly $CD8^+$ cytotoxic lymphocytes. The absence of $CD4^+$ lymphocytes in the CNS, either early or late after infection with SIV, and the inability of $CD8^+$ lymphocytes to support SIV or HIV replication render it unlikely that lymphocytes play a major role in neuroinvasion by SIV or HIV.

In conclusion, we demonstrated that a significant increase in the density of perivascular macrophages coincides with viral neuroinvasion and intrathecal immune activation at 2 weeks postinoculation in animals inoculated with pathogenic isolates of SIV. *In situ* hybridization and combined *in situ* hybridization/immunohistochemistry demonstrate that virus-positive cells characteristic of perivascular macrophages/microglia express monocyte/macrophage-specific antigen. These findings support the Trojan horse theory of SIV/HIV neuroinvasion and suggest that initial viral entry occurs via turnover of perivascular macrophages/microglia and replacement by circulating monocytes. It is likely that this recruitment is a complex process involving chemoattractants as well as leukocyte-endothelial adhesion molecule interactions. While VCAM-1 is important in the development of SIV encephalitis, the role of additional chemoattractants and other adhesion molecules early in infection during initial viral neuroinvasion needs to be examined.

Materials and methods

Animals and virus

Twenty-four male rhesus macaques, 22 to 41 months of age, were obtained from the Type D retrovirus- and SIV-free colony at the California Regional Primate Research Center. Animals were randomly divided into three groups of eight and housed in accordance with standards determined by the American Association for Accreditation of Laboratory Animal Care. In addition, four healthy, age-matched animals were sacrificed as normal controls. Investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

Prior to inoculation all animals tested negative for antibodies to HIV-2, SIV, Type D retrovirus, and simian T-cell leukemia virus type 1 (STLV-1). Each group was inoculated intravenously with a cell-free stock of uncloned SIV_{mac}, SIV_{mac239} or $SIV_{mac1A11}$. Group One was inoculated with $10^{2.5}$ 50% TCID of uncloned SIV_{mac} (derived from biologic isolate SIV_{mac251}, provided by R Desrosiers, New England Regional Primate Research Center). Group Two was inoculated with 10^4 50% TCID of the pathogenic clone SIV_{mac239} (Kestler *et al*, 1990). Group Three was inoculated with 10^4 50% TCID of the nonpathogenic clone $SIV_{mac1A11}$ (Marthas *et al*, 1989; 1990). The dosage of $SIV_{mac1A11}$, equal to that of SIV_{mac239}, was chosen on the basis of past ability to induce viremia in all animals inoculated. The dosages used of SIV_{mac} and SIV_{mac239} routinely cause disease within 6 to 12 months (Marthas *et al*, 1993). Biologic and genetic comparisons of these pathogenic and nonpathogenic clones have been described in detail (Luciw *et al*, 1992). Results of viral isolation and examination of humoral immune response, intrathecal immune response, and viral distribution have been published previously (Lackner *et al*, 1994; Smith *et al*, 1995).

Virus Isolation

Peripheral blood and CSF were collected immediately before inoculation and at 1, 2, 4, 8, 11, 13, 21 and 23 weeks after inoculation. Virus was isolated from CSF and blood by cocultivation with CEM × 174 cells as previously described (Lackner *et al*, 1994). Culture supernatants were tested biweekly for SIV by p27 capture EIA, (Coulter Immunology, Hialeah, Florida). To be considered positive, culture supernatants were required to yield at least 10 ng/ml of SIV p27 at two consecutive time points. Cultures were discarded if virus-negative after 8 weeks.

Quinolinic Acid

Quinolinic acid (QUIN) in CSF was quantified by electron-negative chemical ionization gas chromatography/mass spectroscopy as previously described (Heyes and Markey, 1988a,b). Briefly, CSF QUIN and QUIN standards, 3 to 150 pmol, diluted in deionized water, were mixed with 300 μl of deionized water containing 30 pmol of [^{18}O]-QUIN as an internal standard. Samples were freeze-dried

overnight and esterified. The dihexafluoroisopropanol esters were extracted in heptane, and analysed using gas chromatography with a Hewlett Packard quadrupole mass filter (HP5988 Hewlett-Packard, Palo Alto, CA). Endogenous and [^{18}O]QUIN were measured as molecular ions. QUIN levels were log transformed and subjected to two-way ANOVA comparing effects of virus and time postinoculation.

Tissue collection, histopathology and immunohistochemistry

Two animals from each group were sacrificed at 2, 8, 13 and 23 weeks postinoculation by intravenous injection of sodium pentobarbital. Representative CNS tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μ and stained with hematoxylin and eosin by routine histologic techniques. Parallel sections were subjected to *in situ* hybridization and immunohistochemistry. Frozen tissues were embedded in optimum cutting temperature compound (OCT, Miles Inc., Elkhart, IN), frozen in freon cooled in liquid nitrogen, sectioned on a cryomicrotome and stored at -70°C until further processing.

To identify perivascular macrophages/microglia we performed immunohistochemistry on paraffin sections with monoclonal antibody HAM56 (Dako, Carpinteria, CA) as previously described (Lackner *et al*, 1991). This method was found to be superior to frozen sections immunostained with EBM-11 (CD68, Dako) for the purpose of enumerating labeled cells. Monoclonal antibody EBM-11 was not used on formalin-fixed tissues because of inconsistent results. In macaques, the antibody HAM56 recognizes an uncharacterized intracytoplasmic antigen present in perivascular macrophages/microglia and activated parenchymal microglia. Briefly, sections of frontal cortex were deparaffinized and endogenous peroxidase was blocked by immersion in 0.3% hydrogen peroxide in methanol for 5 min. Antigen was unmasked by a 25 min digestion with proteinase K (Dako) diluted 1:50 in 0.05 M Tris-HC1, pH 7.58. Nonspecific binding was blocked with 10% normal horse serum for 5 min. The primary antibody, HAM56, was diluted 1:100 and incubated overnight at 4°C followed by incubation with biotinylated horse anti-mouse antibody, then avidin-biotin-conjugated horseradish peroxidase complex (Vector Laboratories, Inc., Burlingame, CA). Diaminobenzidine (DAB) was used as the chromogen. Sections were lightly counterstained with Mayer's hematoxylin. To examine the role of VCAM-1 in leukocyte recruitment to the CNS, we performed immunohistochemistry on frozen sections of cerebrum using monoclonal antibody 2G7 (Otsuka America Pharmaceutical, Inc., Rockville, MD) as previously described (Sasseville *et al*, 1992). The relative intensity of VCAM-1 expression was scored blindly as previously described (Silber *et al*, 1994).

Quantitation of perivascular macrophages/microglia

Sections of frontal cortex immunostained with HAM56 were examined to assess numbers of perivascular macrophages within cortical white matter using a Quantimet 570c image analyzer (Leica, Cambridge, UK). We focused quantitation of perivascular macrophages on cortical white matter because we observed that, while these cells are diffusely distributed along blood vessels throughout the CNS, virus and parenchymal inflammatory cell infiltrates are (1) intimately associated with vessels and (2) more common in white matter than cortical or deep grey matter. Ten nonoverlapping 200 × fields of cortical white matter per animal (527,640 μ^2) were examined. All vessel profiles within the 10 fields were measured in greatest length and corresponding perpendicular width (vascular dimensions). Occurrence of each perivascular macrophage was recorded as a single event. For the recording of each event, the intervening vascular dimensions were summed. The frequency of perivascular macrophages/microglia was expressed as a quotient (cells/millimeter of vascular endothelium). The mean, range, and standard error for the number of perivascular macrophages per millimeter of vascular endothelium were calculated for each animal. Differences in mean perivascular macrophage density among postinoculation intervals and viral isolates were examined using ANOVA followed by post hoc testing using Fischer's Protected Least Significant Difference Statistic. (Statview 4.1 statistics software, Abacus Concepts, Inc., Berkeley, CA).

In situ *hybridization and combined* in situ *hybridization/immunohistochemistry*

To examine the distribution of SIV in CNS parenchyma, paraffin-embedded sections of brain adjacent to those used for routine histopathology and immunohistochemistry (HAM56) were examined by *in situ* hybridization using a ^{35}S-labeled DNA probe encompassing the entire *gag, pol* and *env* regions of SIV as previously described (Lackner *et al*, 1994). Controls included SIV-infected and uninfected T cells (HUT78), matched tissues from uninfected macaques and probe derived from nick-translated plasmid vector pSP64.

On selected brain sections, combined *in situ* hybridization/immunohistochemistry was performed as previously described (Heise *et al*, 1994). Briefly, *in situ* hybridization for SIV was performed followed by immunohistochemistry for HAM56 immediately after posthybridization washes as described above except that 3-amino-9-ethylcarbazole (red precipitate) was used as the chromogen. Slides were then rinsed and air-dried before coating with emulsion and then exposed for 1 to 2 weeks at 4°C.

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References

Andersson P, Perry V, Gordon S (1992a). The acute inflammatory response to lipopolysaccharide in the CNS parenchyma differs from that in other body tissues. *Neuroscience* **48:** 169–186.

Andersson P, Perry V, Gordon S (1992b). Intracerebral injection of proinflammatory cytokines or leukocyte chemotaxins induces minimal myelomonocytic cell recruitment to the parenchyma of the central nervous system. *J Exp Med* **176:** 255–259.

Boche D, Gray F, Chakrabarti L, Hurtrel M, Montagnier L, Hurtrel B (1995). Low susceptibility of resident microglia to simian immunodeficiency virus replication during the early stages of infection. *Neuropath Applied Neurobiol* **21:** 535–539.

Brinkmann R, Schwinn A, Müller J, Stahl-Henning C, Coulibaly C, Hunsmann G, Czub S, Rethwilm A, Dörries R, Ter Meulen V (1993). In vitro and in vivo infection of rhesus monkey microglial cells by simian immunodeficiency virus. *Virology* **195:** 561–568.

Brinkmann R, Schwinn A, Narayan O, Zink C, Kreth W, Roggendorf W, Dorries R, Schwender S, Imrich H, ter Meulen V (1992). Human immunodeficiency virus infection in microglia: Correlation between cells infected in the brain and cells cultured from infectious brain tissue. *Ann Neurol* **31:** 349–360.

Chakrabarti L, Hurtrel M, Maire M, 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.

Chiodi F, Albert J, Olausson E, Norkrans G, Hagberg L, Sönnerborg A, Åsjö B, Fenyö E (1988). Isolation frequency of human immunodeficiency virus from cerebrospinal fluid and blood of patients with varying severity of HIV infection. *AIDS Res Human Retrov* **4:** 351–358.

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

Genis P, Jett M, Bernton EW, Boyle T, Gelbard HA, Dzenko K, Keane RW, Resnick L, Mizrachi Y, Volsky DJ, Epstein LG, Gendelman HE (1992). Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interactions: implications for the neuropathogenesis of HIV disease. *J Exp Med* **176:** 1703–1718.

Heise C, Miller C, Lackner A, Dandekar S (1994). Primary acute simian immunodeficiency virus infection of intestinal lymphoid tissue is associated with gastrointestinal dysfunction. *J Infect Dis* **169:** 1116–1120.

Heyes MP, Brew BJ, Martin A, Price RW, Salazar AM, Sidtis JJ, Yergey JA, Mouradian MM, Sadler AE, Keilp J, Rubinow D, Markey SP (1991). Quinolinic acid in cerebrospinal fluid and serum in HIV-1 infection: Relationship to clinical and neurological status. *Ann Neurol* **29:** 202–209.

Heyes MP, Markey SP (1988a). (^{18}0)quinolinic acid: its esterification without back exchange for use as internal standard in the quantification of brain and CSF quinolinic acid. *Biomed Environ Mass Spectrom* **15:** 291–293.

Heyes MP, Markey SP (1988b). Quantification of quinolinic acid in rat brain, whole blood and plasma by gas chromatography and negative chemical ionization mass spectroscopy: effects of systemic 1-tryptophan administration on brain and whole blood quinolinic acid concentrations. *Anal Biochem* **174:** 349–359.

Heyes MP, Saito K, Crowley JS, Davis LE, Demitrack MA, Der M, Dilling LA, Elia J, Kruesi MJP, Lackner A, Larsen SA, Lee K, Leonard HL, Markey SP, Martin A, Milstein S, Mouradian MM, Pranzatelli MR, Quearry BJ, Salazar A, Smith M, Strauss SE, Sunderland T, Swedo SW, Tourtellotte WW (1992). Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. *Brain* **115:** 1249–1273.

Hickey WF, Hsu BL, Kimura H (1991). T-lymphocyte entry into the central nervous system. *J of Neuroscience Res* **28:** 254–260.

Hickey WF, Kimura H (1988). Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* **239:** 290–292.

Ho DD, Rota TR, Schooley RT, Kaplan JC, Allan JD, Groopman JE, Resnick L, Felsenstein D, Andrews CA, Hirsch MS (1985). Isolation of HTLV-III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome. *N Engl J Med* **313:** 1493–1497.

Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, Regier D, Sehgal P, Daniel M, King N, Desrosiers R (1990). Induction of AIDS in rhesus monkeys by molecularly cloned immunodeficiency virus. *Science* **239:** 586–591.

Koch A, Kunkel S, Harlow L, Mazarakis D, Haines G, Brdick M, Pope R, Strieter R (1994). Macrophage inflammatory protein-1a: a novel chemotactic cytokine for macrophages in rheumatoid arthritis. *Journal of Clinical Investigation* **93:** 921–928.

Koenig S, Gendelman HE, Orenstein JM, Dal Canto MC, Pezeshkpour GH, Yungbluth P, Janotta F, Aksamit A, Martin M, Fauci AS (1986). Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233:** 1089–1093.

Lackner A, Vogel P, Ramos R, Kluge J, Marthas M (1994). Early events in tissues during infection with pathogenic (SIVmac239) and nonpathogenic (SIVmac1A11) molecular clones of SIV. *Am J Pathol* **145:** 428–439.

Lackner AA, Smith MO, Munn RJ, Martfeld DJ, Gardner MB, Marx PA, Dandekar S (1991). Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *Am J Pathol* **139:** 609–621.

Lassman H, Schmied M, Vass K, Hickey W (1993). Bone marrow derived elements and resident microglia in brain inflammation. *Glia* **7:** 19–24.

Lawson L, Perry V, Gordon S (1992). Turnover of resident microglia in the normal adult mouse brain. *Neuroscience* **48:** 405–415.

Ljubisa V, de Cunha A, Tyor WR (1994). Cytokine expression and pathogenesis in AIDS Brain. In: *HIV, AIDS, and the Brain.* Price RW and Perry SW (eds). Raven Press: New York, pp 203–222.

Luciw P, Shaw K, Unger R, Planelles V, Stout M, Lackner J, Pratt-Lowe E, Leung N, Banapour B, Marthas M (1992). Genetic and biological comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). *AIDS Res Hum Retroviruses* **8:** 395–400.

Marthas ML, Banapour B, Sutjipto S, Siegel ME, Marx PA, Gardner MB, Pedersen NC, Luciw PA (1989). Rhesus macaques inoculated with molecularly cloned simian immunodeficiency virus. *J Med Primatol* **18:** 311–319.

Marthas ML, Ramos RA, Lohman BL, Van Rompay KKA, Unger RE, Miller CJ, Banapour B, Pedersen N, Luciw PA (1993). Viral determinants of simian immunodeficiency virus (SIV) virulence in rhesus macaques assessed by using attenuated and pathogenic molecular clones of SIVmac. *J Virol* **67:** 6047–6055.

Marthas ML, Sutjipto S, Higgins J, Lohman B, Torten J, Luciw PA, Marx PA, Pedersen NC (1990). Immunization with a live, attenuated simian immunodeficiency virus (SIV) prevents early disease but not infection in rhesus macaques challenged with pathogenic SIV. *J Virol* **64:** 3694–3700.

Nottet H, Persidsky Y, Sasseville V, Nukuna A, Bock P, Zhai Q, Sharer L, McComb R, Swindells S, Soderland C, Gendelman H (1996). Mechanisms for the transendothelial migration of HIV-1-infected monocytes into brain. *J Immunol* **156:** 1284–1295.

Peluso R, Haase A, Stowring L, Edwards M, Ventura P (1985). A trojan horse mechanism for the spread of visna virus in monocytes. *Virology* **147:** 231–236.

Perry VH (1994). *Macrophages and the Nervous System* R.G. Landes Co.: Austin, pp. 123.

Pumarola-Sune T, Navia B, Cordon-Cardo C, Eun-Sook C, Price R (1987). HIV antigen in the brains of patients with the AIDS dementia complex. *Ann Neurol* **21:** 490–496.

Reimann KA, Tenner-Racz K, Racz P, Montefiori DC, Yasutomi Y, Lin W, Ransil BJ, Letvin NL (1994). Immunopathogenic events in acute infection of rhesus monkeys with simian immunodeficiency virus of macaques. *J Virol* **68:** 2362–2370.

Sasseville VG, Lane JH, Walsh D, Ringler DJ, Lackner AA (1995). VCAM-1 expression and leukocyte trafficking to the CNS occur early in infection with pathogenic isolates of SIV. *J Med Primatol* **24:** 123–131.

Sasseville VG, Newman W, Brodie SJ, Hesterberg P, Pauley DR, Ringler DJ (1994). Monocyte adhesion to endothelium in simian immunodeficiency virus-induced AIDS encephalitis is mediated by vascular cell adhesion molecule-1/$\alpha 4\beta 1$ integrin interactions. *Am J Pathol* **144:** 27–40.

Sasseville VG, Newman WA, Lackner AA, Smith MO, Lausen N, Beall D, Ringler DJ (1992). Elevated vascular cell adhesion molecule-1 in AIDS induced by simian immunodeficiency virus. *Am J Pathol* **141:** 1021–1030.

Schmidmayerova H, Nottet H, Nuovo G, Raabe T, Flanagan C, Dubrovsky L, Gendelman H, Cerami A, Bukrinsky M, Sherry B (1995). Human immunodeficiency virus type 1 infection alters chemokine β peptide expression in human monocytes: Implications for recruitment of leukocytes into brain and lymph nodes. *Proc Natl Acad Sci* **93:** 700–704.

Silber A, Newman W, Sasseville VG, Pauley D, Beall D, Walsh DG, Ringler DJ (1994). Recruitment of lymphocytes during cutaneous delayed hypersensitivity in nonhuman primates is dependent on E-selectin and vascular cell adhesion molecule 1. *J Clin Invest* **93:** 1554–1563.

Smith MO, Heyes MP, Lackner AA (1995). Early intrathecal events in rhesus macaques (*Macaca mulatta*) infected with pathogenic or nonpathogenic molecular clones of simian immunodeficiency virus. *Lab Invest* **72:** 547–558.

Taub D, Conlon K, Lloyd A, Oppenheim J, Kelvin D (1993). Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1a and MIP-1b. *Science* **260:** 355–358.

Vazeux R, Brousse N, Jarry A, Henin D, Marche C, Vedrenne C, Mikol J, Wolff M, Michon C, Rozenbaum W, Bureau JF, Montagnier L, Brahic M (1987). AIDS subacute encephalitis: Identification of HIV-infected cells. *Am J Pathol* **126:** 403–410.

VonHerrath M, Oldstone M, Fox H (1995). SIV-Specific CTL in Cerebrospinal fluid and Brains of SIV-infected Rhesus Macaques. *J of Immunol* **154:** 5582–5589.

Watry DTL, Streb M, Fox H (1995). Transfer of neuropathogenic simian immunodeficiency virus with naturally infected microglia. *American Journal of Pathology* **146:** 914–923.

Zhang J, Martin LN, Watson EA, Montelaro RC, West M, Epstein L, Murphey-Corb M (1988). Simian Immunodeficiency Virus/Delta-induced immunodeficiency disease in rhesus monkeys: Relation of antibody response and antigenemia. *J Infect Dis* **158:** 1277–1286.