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Identification of T lymphocytes in simian immunodeficiency virus encephalitis: Distribution of CD8$^+$ T cells in association with central nervous system vessels and virus

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T lymphocytes are found within brains infected with human immunodeficiency virus (HIV) or simian immunodeficiency virus (SIV) where they are a minor, but consistently identified, population. However, little analysis of their phenotypes has been done, and questions concerning whether or not they are viral antigen specific has not been thoroughly examined. We investigated the central nervous system (CNS) of SIV-infected rhesus macaques to identify T-lymphocyte subsets in relation to virus-infected cells and brain microvessels. We have found that a sensitive antigen-retrieval technique greatly enhanced immunohistochemical detection of CD4$^+$ and CD8$^+$ T lymphocytes in control studies. In encephalitic brains of SIV-infected monkeys with acquired immunodeficiency syndrome (AIDS), we found a significant accumulation of CD8$^+$ T lymphocytes but little-to-no accumulation of CD4$^+$ T lymphocytes. CD4$^+$ cells, when detected, were mostly monocyte/macrophages closely associated with CNS vessels. Using a combination of *in situ* hybridization for SIV RNA, and immunohistochemistry for CD8$^+$ T lymphocytes and/or Glut-1 for endothelial cells on brain microvessels, we found CD8$^+$ T lymphocytes with an angiocentric distribution often adjacent to virus-infected cells. In the CNS of animals with SIV encephalitis, there was a trend of CD8$^+$ T lymphocytes that were not directly juxtaposed with CNS vessels. These data suggest that in brains of SIV-infected monkeys and HIV-infected humans, CD8$^+$ T lymphocytes traffic to and are retained in the CNS in an angiocentric and possibly antigen-specific manner. *Journal of NeuroVirology* (2004) **10,** 315–325.

Keywords: brain; perivascular macrophages; SIV encephalitis; T lymphocytes

Introduction

The central nervous system (CNS) has long been considered as an "immune-privileged" site, devoid of cellular elements of the immune system (Broadwell *et al*, 1990; Streilein, 1995). Evidence of T lymphocytes and monocyte/macrophages infiltrating the CNS under noninflammatory conditions and inflammation with viral infection and autoimmune disease showed that the CNS is partially immune privileged

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(Hickey, 1991; Griffin *et al*, 1992; Williams and Hickey, 1995). Recent studies underscore the potential importance of these cells, especially the $CD8^+$ T-lymphocyte subset, in CNS immunity and viral infection, suggesting a need for the reevaluation of their role in CNS diseases (Katsetos *et al*, 1999; Babbe *et al*, 2000; Lellouch-Tubiana *et al*, 2000; Bauer *et al*, 2001; Bien *et al*, 2002; Togo *et al*, 2002; Petito *et al*, 2003).

Involvement of T lymphocytes in the neuropathogenesis associated with human immunodeficiency virus (HIV) and its close relative simian immunodeficiency virus (SIV) has been suspected for many years. Earlier studies investigating HIV-infected humans and SIV-infected rhesus macaques identified infiltrating T lymphocytes in the brain (Vazeux *et al*, 1987; Parravicini *et al*, 1989; Porwit *et al*, 1989; Lackner *et al*, 1991; Bell *et al*, 1993; Weidenheim *et al*, 1993; Boche *et al*, 1999). However, this group of immune cells has received little attention, in part, due to the lack of antibodies that label CD4 and CD8 T-cell antigens in paraffin-embedded tissues.

In the present study, we examined the brains of SIV-infected rhesus macaques using human CD4 and CD8 monoclonal antibodies (mAbs) and an antigen-retrieval technique for optimal treatment of paraffin-embedded tissues to increase the immunohistochemical labeling (Mason *et al*, 1992; Williamson *et al*, 1998). Using this technique, we can reliably visualize T lymphocytes in paraffin-embedded simian tissues and study T-lymphocyte subsets in the monkey brain. We employed these antibodies and combined *in situ* hybridization for SIV RNA and immunohistochemistry for CNS endothelial cells on microvessels to investigate the distribution of T lymphocytes in SIV encephalitis (SIVE) and to study their possible traffic and retention. We found both $CD4^+$ and $CD8^+$ T lymphocytes in CNS tissues, with a preponderance of $CD8^+$ T lymphocytes in perivascular cuffs and the parenchyma. When $CD4^+$ T lymphocytes were found, they were in the meninges and choroid plexus. Within the CNS parenchyma, the majority of CD4 antigens were found on perivascular macrophages and multinucleated giant cells (MNGCs) and scattered parenchymal microglia. Multilabel immunohistochemistry for $CD8^+$ lymphocytes, Glut-1–positive CNS endothelium coupled with *in situ* hybridization for SIV RNA showed that $CD8^+$ lymphocytes in the CNS were associated with CNS vessels and virus-infected cells. A few scattered $CD8^+$ T cells were found in the parenchyma, primarily in the white matter, and were often also found associated with virus-infected cells. These observations underscore the role of $CD8^+$ T lymphocytes in the CNS of viral encephalitis and suggest that these cells traffic to and are retained in the CNS possibly in an antigen-specific manner.

Results

We first evaluated the effectiveness of anti-human CD4 (clone 1F6) and CD8 (clone 1A5) mAbs to detect CD4 and CD8 antigens in paraffin-embedded monkey and human tissues. Initially, paraffin sections of spleen and lymph node from normal and SIV-infected monkeys and normal human tonsil were tested with different antigen-retrieval techniques including proteinase K digestion, microwave pretreatment with 1 mM EDTA (pH 8.0) or 0.01 M sodium citrate buffer (pH 6.0), and pressure cooker treatment with Trilogy (EDTA solution). No distinct positive staining was observed without antigen retrieval on any of the paraffin-embedded tissues examined (data not shown). CD4- and CD8-stained cells were readily and consistently detected only after pressure cooker pretreatment with Trilogy (Figures 1, 2). In addition to analysis of $CD4^+$ and $CD8^+$ T cells in lymph node, spleen, and tonsil, the pressure cooker and Trilogy antigen-retrieval methods also resulted in intense and specific staining of $CD8^+$ and $CD4^+$ lymphocytes and $CD4^+$ CNS macrophages within SIV-infected brain tissues (Figures 1, 2). Most cells expressing CD4 antigens in SIV-infected brain had histiocytic, rather than lymphocytic, morphological characteristics. These $CD4^+$ cells included perivascular macrophages, MNGCs, and scattered process-bearing cells consistent with parenchymal microglia (Figure 2). Because different antigen-retrieval methods can generate neo-antigens resulting in nonspecific staining, we compared the results of immunohistochemistry on lymph node, spleen, and brain of formalin-fixed, paraffin-embedded tissues, with immunohistochemistry on frozen tissue sections from the same organs of the same animals. In all cases, these results were comparable with regard to antibody sensitivity, and the distribution and morphology of CD4- and CD8-immunoreactive cells, although the morphology of lymphoid tissues in formalin-fixed tissues was superior to that of frozen tissue sections (Figure 1).

To investigate the frequency and distribution of T lymphocytes in brains of SIV-infected rhesus macaques, we studied animals with acquired immunodeficiency syndrome (AIDS) and SIVE (SIVE) as well as SIV-infected animals with AIDS and without encephalitis (SIVnoE), SIV-infected animals sacrificed at peak viremia (14 days post infection [d.p.i.]; viremic), and normal uninfected controls (Table 1). To distinguish the $CD4^+$ and $CD8^+$ lymphocytes from brain macrophages and natural killer (NK) cells that also express CD4 and CD8 respectively, but not CD3, we performed double-label experiments with an anti-CD3 polyclonal antibody and anti-CD4 or anti-CD8 (Figures 2, 3). In addition, we used an anti–Glut-1 polyclonal antibody, which labels endothelial cells in CNS microvessels, to assess the association of T cells with CNS vessels. CD4

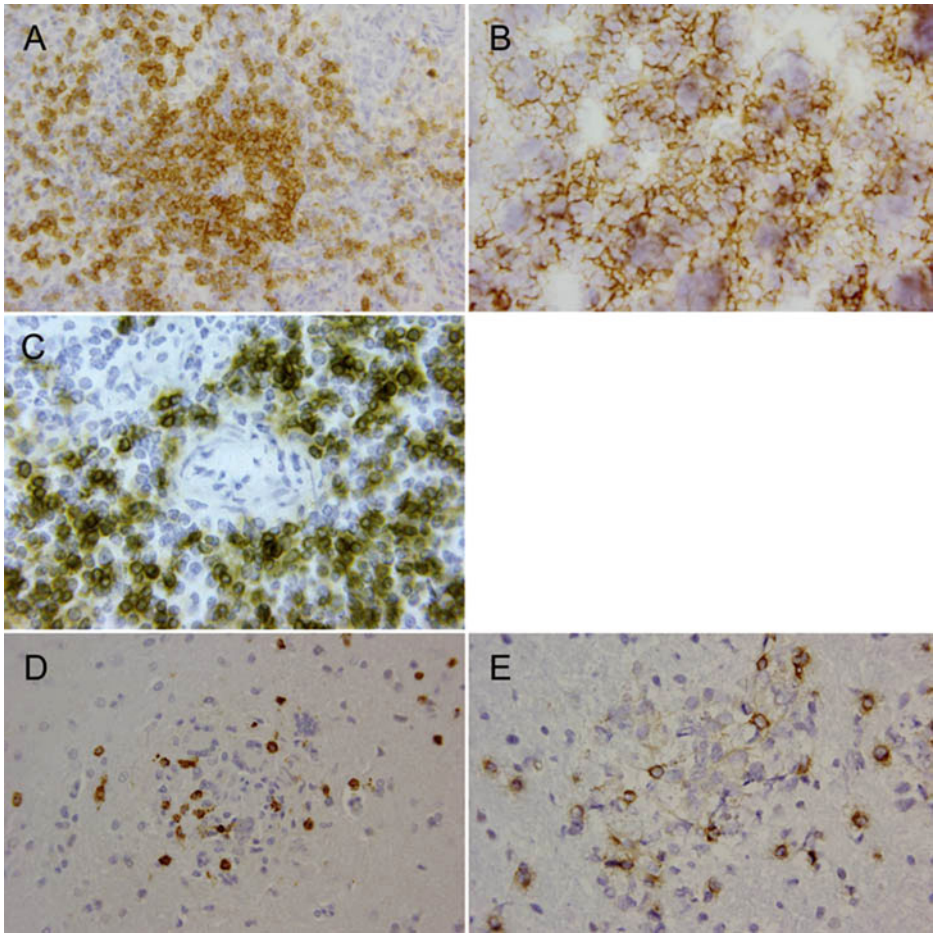

Figure 1 Immunohistochemical staining of SIV-infected rhesus monkey tissues with anti-CD8 mAb, clone 1A5; comparing paraffin (**A**, **C**, **D**) and cryostat sections (**B**, **E**). (**A**) CD8 reactivity within the paracortex of a lymph node using paraffin-embedded tissues. (**B**) CD8 reactivity within the paracortical area of a lymph node, demonstrated on fresh-frozen lymph node. *Note*: Superior morphology of $CD8^+$ T lymphocytes in paraffin-embedded sections (*left panel*) than frozen sections (*right panel*). (**C**) CD8 reactivity on T lymphocytes surrounding an arteriole within the splenic white pulp demonstrated using paraffin-embedded tissue. (**D**) CD8 reactivity on lymphocytes within an SIVE lesion demonstrated using paraffin-embedded CNS tissue. (**E**) CD8 reactivity within an SIVE lesion using fresh frozen CNS tissues. Data presented here are representative of two SIV-infected rhesus macaques with AIDS. 400× magnification.

immunoreactivity in animals with AIDS and SIVE was found in the meninges, perivascular cuffs, and scattered cells in the parenchyma (Figure 2). The majority of these cells were associated with Glut-1–positive CNS endothelial cells. The morphology of the cells expressing CD4 antigens was consistent with CNS macrophages. Double-label immunohistochemistry with CD3 and CD4 antibodies showed little-to-no colocalization of CD3 with the $CD4^+$ cells confirming that very few if any $CD4^+$ T lymphocytes are present in the CNS parenchyma of these animals (Figure 2C). When $CD4^+$ T lymphocytes were detected, they were mainly confined to the meninges and choroid plexus. In noninfected control and viremic animals, CD4-stained cells were not detected in the parenchyma; however, $CD4^+$ macrophages and rare $CD4^+$ lymphocytes were found in the meninges and choroid plexus (data not shown). All of the SIVE cases examined contained variable numbers of CD8-positive cells in the perivascular cuffs and the brain parenchyma (Figure 3) and had significantly higher number of $CD8^+$ T lymphocytes (mean ± SEM, 87.4 ± 19.5 cells/50 mm^2) than control (9.4 ± 2.0), viremic (9.3 ± 2.6), and SIVnoE cases (13.8 ± 3.1) ($P < .01$, Tukey HSD test; Table 1; Figure 4A). These $CD8^+$ cells displayed intense membrane staining and were lymphoid cells based on morphology. In noninfected control, viremic, and SIVnoE animals, a few scattered CD8-positive cells were found exclusively confined to the meninges and perivascular cuffs. Double-label immunohistochemistry for CD3 (pan-T-cell marker) followed by CD8 demonstrated that the majority of CD3 T cells in the brain parenchyma of all cases examined in this study were also CD8-positive T lymphocytes (Figure 3C, Table 1). When these $CD8^+$ T lymphocytes were characterized for their distribution relative to Glut-1–positive CNS vessels, we found that

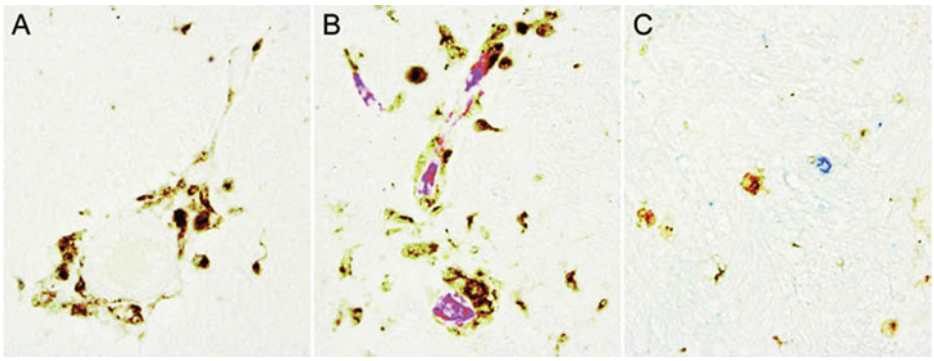

Figure 2 Representative immunohistochemistry of paraffin-embedded CNS tissues from animals with AIDS and SIVE using anti-CD4 mAb, clone 1F6. (**A**) Single-color immunohistochemical detection of CD4 reactivity on cells in the perivascular cuffs (brown reaction product). (**B**) Double-label immunohistochemistry showing the association of CD4-stained cells (*brown*) with CNS vessels labeled with anti–Glut-1 antibody (*red*). (**C**) Double-label immunohistochemistry with anti-CD4 mAb (*brown*) and polyclonal anti-CD3 antibody (*blue*) showing no colocalization of CD4-stained cells with $CD3^+$ T lymphocytes, indicating these $CD4^+$ cells are primarily perivascular macrophages. Data presented here are representative of immunohistochemical staining of nine rhesus macaques with AIDS and SIVE. 400× magnification.

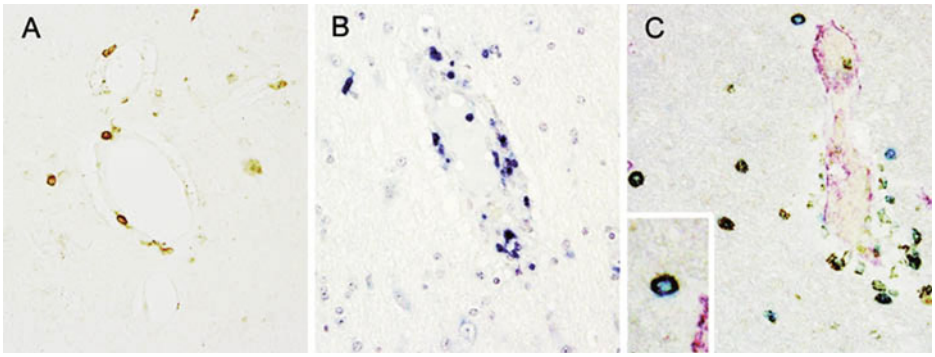

Figure 3 Immunophenotype and accumulation of $CD8^+$ cells. (**A**) Single-color immunohistochemical detection of $CD8^+$ cells near CNS vessels (*brown*). (**B**) Single-color immunohistochemical detection of $CD3^+$ T lymphocyte (*blue*). (**C**) Double-label immunohistochemistry for CD3 followed by CD8 show that essentially all of the $CD8^+$ cells are $CD3^+$ T lymphocytes (colocalized brown-blue reaction product with a black rim). Glut-1 (red). *Inset*: Representative double-stained ($CD3^+/CD8^+$) small round lymphocyte. Data presented here are representative of immunohistochemical staining of rhesus macaques with AIDS and SIVE ($n = 9$). 400× magnification.

in the SIVE group, $CD8^+$ cells were more diffusely involved in the white matter than in the non-SIVE groups where infiltration of the CNS by these cells was tightly confined to the perivascular cuffs. The significantly reduced percentage of $CD8^+$ T lymphocytes associated with vessels in SIVE ($P < .01$) may represent the extravasation of these lymphocytes into the perivascular space and the subsequent infiltration into the brain parenchyma (Table 1, Figure 4B). There is also a trend towards inverse correlation between the number of total $CD8^+$ T cells and the percentage of $CD8^+$ T cells associated with vessels ($r^2 = .42$, $P < .0001$; Figure 5).

Next we performed *in situ* hybridization for viral nucleic acids on monkey tissues followed by immunohistochemistry for $CD8^+$ T lymphocytes. A combined *in situ* hybridization for SIV RNA and immunohistochemistry for CD8 antigen was performed to visualize virus-infected cells and $CD8^+$ T lymphocytes in CNS of SIV-infected monkeys with AIDS and SIVE (Figure 6). *In situ* hybridization for SIV RNA showed many SIV-infected cells, including perivascular macrophages, MNGCs, and macrophages in SIVE lesions, as has been reported previously (Chakrabarti *et al*, 1991; Hurtrel *et al*, 1991; Lane *et al*, 1996; Reinhart *et al*, 1997; Williams *et al*, 2001). Often, these cells were associated with Glut-1–positive CNS endothelial cells on microvessels. Double-label *in situ* hybridization for SIV RNA and immunohistochemistry for $CD8^+$ T lymphocytes demonstrated an accumulation of $CD8^+$ T lymphocytes within SIVE lesions that consisted of SIV RNA–positive cells (Figure 6). The majority of $CD8^+$ T lymphocytes were located within or around these SIVE lesions (mean 66.2%) (Table 1, Figure 6). In addition to these CD8 lymphocytes, a few scattered $CD8^+$ T lymphocytes also were found within the CNS parenchyma. When found, these cells were predominantly in the white matter. Interestingly, in several instances we found individual CD8 T lymphocytes in the white matter parenchyma that were in contact with SIV RNA–positive cells, suggesting these cells,

Table 1 Number and distribution of $CD8^+$ T lymphocytes in the cerebrums[a] of SIV-infected macaques

Case	*$CD8^+/CD3^+$ (%)*[b]	*$CD8^+$ cells per 50 mm^2 CNS tissue*	*$CD8^+$ cells in with vessels association (% of all $CD8^+$ cells)*[c]	*$CD8^+$ cells in proximity to infected cells (% of all $CD8^+$ cells)*[c]
Control		(9.4 ± 2.0)	(89.5 ± 3.6)	
01072a	96.6	13.0	93.5	n.a.
01072b	93.9	20.6	94.9	n.a.
01072c	n.d.	11.8	n.d.	n.a.
03084a	100	6.0	88.9	n.a.
03084b	n.d.	13.8	71.2	n.a.
03084c	n.d.	6.1	89.3	n.a.
03084d	n.d.	10.0	78.5	n.a.
03393a	100	1.4	100	n.a.
03393b	100	2.2	100	n.a.
Viremic		(9.3 ± 2.6)	(94.5 ± 2.3)	
99493	97.1	18.5	91.9	n.a.
99513	97.2	12.0	89.7	n.a.
01409	100	5.2	90.9	n.a.
01531	77.8	4.7	100	n.a.
01532	91.7	6.3	100	n.a.
SIVnoE		(13.8 ± 3.1)	(87.3 ± 3.2)	
95212a	88.2	21.9	88.2	n.a.
95212b	86.2	25.9	90.4	n.a.
97248	100	1.5	100	n.a.
04052a	100	6.5	100	n.a.
04052b	n.d.	8.0	100	n.a.
04055	84.4	10.8	71.1	n.a.
04060a	98.0	13.5	84.7	n.a.
04060b	n.d.	36.9	83.2	n.a.
04073a	100	7.7	69.2	n.a.
04073b	n.d.	9.8	88.0	n.a.
04080	n.d.	9.7	85.7	n.a.
SIVE		(87.4 ± 19.5)*	(68.8 ± 3.2)*	(66.2 ± 2.9)
92620	98.2	60.3	66.7	78.2
95346	n.d.	203.7	n.d.	57.9
97076	n.d.	66.3	n.d.	56.5
99732	n.d.	86.1	n.d.	65.9
99750a	91.1	28.5	70.5	79.1
99750b	90.9	20.5	75.4	68.6
01031a	89.9	92.8	71.3	73.5
01031b	91.4	93.9	70.6	69.5
01227	91.1	20.0	77.9	63.0
01622	98.6	234.5	58.2	50.2
03762a	n.d.	52.8	66.3	n.d.
03762b	n.d.	89.3	62.8	n.d.

n.a. = not applied because of no infected cell; n.d. = not determined.
[a]Parenchymal and perivascular $CD8^+$ and $CD3^+$ cells were counted, and T cells in the choroid plexus, the meninges, and the lumen of blood vessels were not taken into account.
[b]Calculated in a single section of the cortex in every case with double immunostaining.
[c]$CD8^+$ cells located in close contact with or within a three-lymphocyte distance from vessels or infected cell were counted.
The numbers in parentheses are the mean number of $CD8^+$ cells ± SEM or the mean percentage of $CD8^+$ cells in association with vessels of each group. *$P < .01$ when all pairs of the groups were compared using an ANOVA with Tukey-Kramer HSD test.

similar to CD8 lymphocytes in SIVE lesions, might be SIV antigen specific (Figure 6C, *inset*).

Discussion

$CD4^+$ and $CD8^+$ T lymphocytes play a critical role in immune control of HIV/SIV infection. The precise role of $CD4^+$ and $CD8^+$ T cells and their antigen-specific traffic and retention in the CNS of HIV-infected patients have not been extensively examined. Although HIV-specific $CD4^+$ lymphocytes have not been found in the cerebrospinal fluid (CSF) of humans, virus-specific $CD8^+$ T-cell clones were derived from the CSF of HIV-infected patients (Sethi *et al*, 1988; Jassoy *et al*, 1992), and $CD8^+$ T cells in CSF and brains of SIV-infected monkeys were shown to be antigen specific (von Herrath *et al*, 1995; Sopper *et al*, 1998; Marcondes *et al*, 2003). To determine whether T cell–mediated immunity occurs in HIVE and the role of lymphocyte subsets in CNS immunity and neuropathogenesis of AIDS, it is essential to characterize the infiltrating T cells of inflammatory lesions in the CNS. In the present study, we have identified subsets of T cells reliably in paraffin-embedded monkey CNS tissues using the pressure-cooking treatment

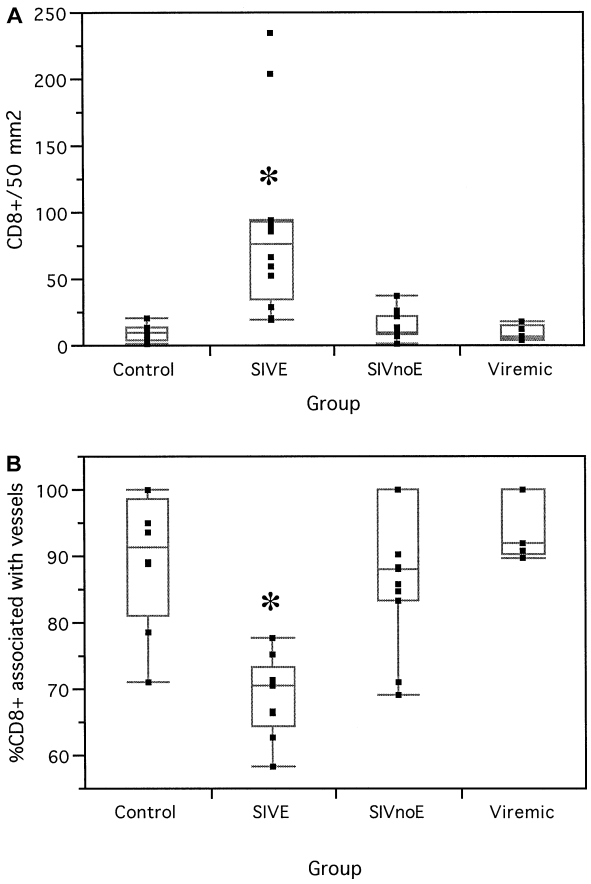

Figure 4 Number of CD8$^+$ T lymphocytes and their association with microvascular endothelium during SIV infection. Differences in total number of CD8$^+$ T lymphocytes (**A**) and percentage of CD8$^+$ T lymphocytes associated with vessels (**B**) between controls and SIV-infected brains. The box plots summarize the distribution of points in each group. The box ends are the 25th and 75th quantiles and the line across the middle is the median value. All pairs of groups were compared using an ANOVA with Tukey-Kramer HSD test for comparisons at the 99% confidence level. *Different when compared with control, viremic, and SIVnoE groups.

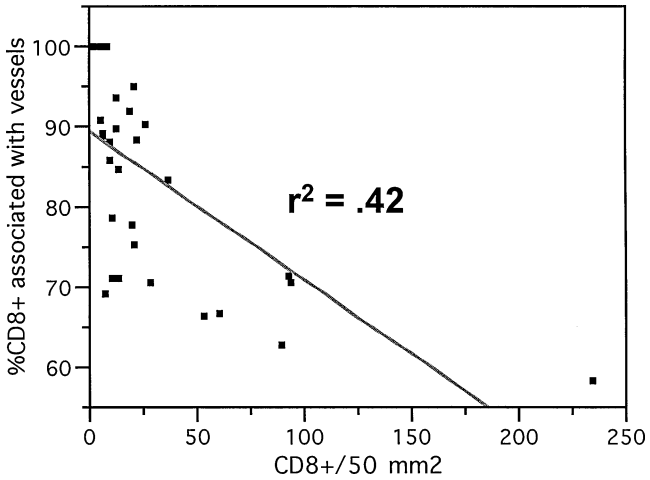

Figure 5 Relationship between the number of total CD8$^+$ T cells and the percentage of CD8$^+$ T cells associated with blood vessels for all cases examined. Scatter plot and bivariate linear fit show a moderate but significant inverse correlation between two parameters with $r^2 = .42$ ($P < .0001$).

for optimal antigen retrieval and the mAbs specific for CD4- and CD8-positive cells.

SIV-infected monkeys are crucial for studies of HIV neuropathogenesis because simian AIDS closely resembles the human counterpart and because SIV causes virus-associated neurological lesions in monkeys similar to those in patients with AIDS (Lackner, 1994). To define infiltrating T-cell populations in neuropathogenesis, we performed immunohistochemical analyses of the brains of rhesus macaques, using SIV model of neuroAIDS. T lymphocytes in paraffin sections of SIV-infected brains were immunophenotyped with the anti-human CD4 and CD8 clones and a polyclonal antibody to CD3 (pan-T-cell marker) all of which function in rhesus macaque tissues. Then to investigate their distribution in association with CNS vessels and virus, double-label

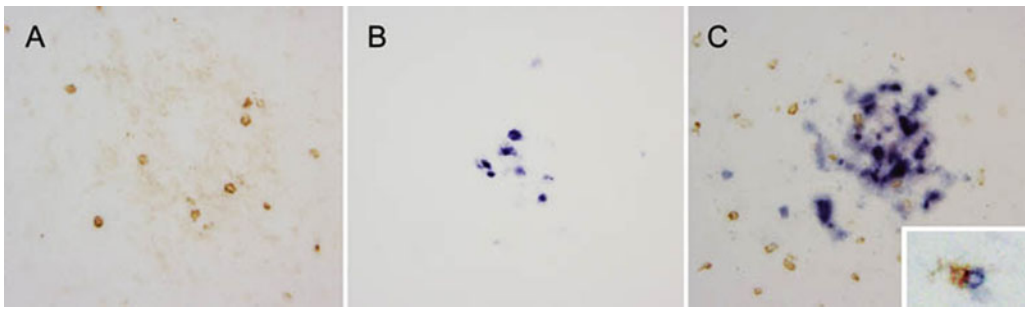

Figure 6 Combined immunohistochemistry for CD8$^+$ T lymphocytes and *in situ* hybridization for virus-infected cells in formalin-fixed, paraffin-embedded CNS tissues of animals with AIDS and SIVE. (**A**) Single-color immunohistochemistry demonstrating CD8 reactivity on lymphocytes within the CNS (*brown*). (**B**) Single-color in situ hybridization for SIV RNA in the CNS (*purple*). (**C**) Double-label *in situ* hybridization for SIV (*purple*) and immunohistochemistry for CD8$^+$ T lymphocytes (*brown*). *Inset*: CD8$^+$ T cell in close contact with SIV RNA–positive (infected) cell. Data presented here for SIV infection are representative of immunohistochemical staining of rhesus macaques with AIDS and SIVE ($n = 9$). 400× magnification.

immunohistochemistry for CD8 antigens with an endothelial cell marker (Glut-1) and combined immunohistochemistry and *in situ* hybridization for viral nucleic acids were done.

Although we did not observe a significant accumulation of T lymphocytes in nonencephalitic brains including control, viremic, and SIVnoE cases, we found abundant $CD3^+/CD8^+$ T cells in most SIVE cases examined. Although this suggests direct association between SIV neuropathology and the number of $CD8^+$ T lymphocytes, whether the accumulation of $CD8^+$ T lymphocytes in brains of animals with SIVE results from immune response against virus-infected cells and/or contributes further to the development of SIV-induced CNS injury has not been addressed in this study.

$CD8^+$ lymphocytes were not only similar in frequency and distribution to the $CD3^+$ T lymphocytes, but double-label experiments confirmed that $CD3^+CD8^+$ lymphocytes comprise the vast majority of $CD3^+$ T cells regardless of the total number of T cells in the brain parenchyma. This finding generally agrees with previous reports in SIVE and HIVE (Pumarola-Sune*et al*, 1987; Parravicini *et al*, 1989; Porwit *et al*, 1989; Lackner *et al*, 1991; Iwasaki *et al*, 1993; Boche *et al*, 1999). A recent report in humans described CD3, CD45RO, CD4, and CD8 lymphocytes in the CNS of patients with AIDS and HIVE (Petito *et al*, 2003). In this study, CD3 and CD45RO cells were strongly immunoreactive, although both CD4 and CD8 were not. The authors stated that there were fewer CD4- and CD8-positive cells than CD3-positive cells, which might, in part, be attributed to variability between antibodies and technical issues. This report found $CD4^+$ T lymphocytes in the CNS of patients with AIDS and HIVE, in contrast to our study. Our finding of a preponderance of $CD8^+$ T lymphocytes over $CD4^+$ lymphocytes in SIVE lesions is consistent with prior reports in SIV-infected rhesus macaques (Chakrabarti *et al*, 1991; Lackner *et al*, 1991). More recently, a study of HIV-infected patients with and without highly active antiretroviral therapy (HAART), some of whom had dementia, found that all of the $CD3^+$ lymphocytes in the brain tissues were CD8-positive and CD4-negative (Miller *et al*, 2004). Additionally, an angiocentric distribution of $CD3^+$ $CD8^+$ lymphocytes has been described in the CNS of HIV-infected children where such a distribution was thought to contribute to vascular damage (Katsetos *et al*, 1999).

In our study, we found that the $CD8^+$ T lymphocytes were largely associated with CNS vessels in both non-SIVE groups and the SIVE group. In the non-SIVE groups, $CD8^+$ T cells were more tightly confined to the perivascular cuffs than in the SIVE group where infiltration of CNS by these cells extended further to the CNS parenchyma. The distribution of $CD8^+$ T lymphocytes in nonencephalitic brains suggests immune surveillance of perivascular spaces by these T cells in the absence of brain infection but not invasion into the parenchyma. Our data further suggest that SIV brain infection triggers the recruitment of CD8 T lymphocytes into the brain parenchyma, although this study did not directly address the timing of the infiltration of these cells. A significantly reduced percentage of $CD8^+$ T cells associated with vessels in SIVE may be due to increased blood-brain barrier disruption and/or to increased neuroinvasiveness of $CD8^+$ T cells. Whether such neuroinvasiveness is the result of antigen-specific traffic or retention is purely speculative.

The perivascular and parenchymal location of the $CD8^+$ T lymphocytes, as has been reported previously in SIVE and HIVE (Porwit *et al*, 1989; Lackner *et al*, 1991; Petito *et al*, 2003), might contribute to vascular and axonal damage. Such damage might occur to a greater extent if the CD8 lymphocyte receives a secondary stimulus within the CNS resulting from interaction with SIV antigens. Indeed, we found an angiocentric distribution of $CD8^+$ T lymphocytes, many of which were adjacent to virus-infected cells, suggesting they are in association with SIV antigens. Whether the scattered $CD8^+$ T lymphocytes in the white matter are engaged in axonal injury is, although suggestive, uncertain. Certainly, $CD8^+$ T lymphocytes elaborate a range of potentially neurotoxic agents that are elevated in the CNS with HIVE and SIVE (Tyor *et al*, 1992; Kaul *et al*, 2001). This observation with the demonstrated accumulation of β-amyloid precursor protein, considered a marker of severed or damaged axons, underscore the possible importance of $CD8^+$ T lymphocytes in axonal clipping as a mechanisms of neuronal injury (Mankowski *et al*, 2002b). Both mechanisms may be particularly important in the case of HIV and SIV following our observation of $CD8^+$ T lymphocytes in proximity to SIV RNA–positive cells. HIV/SIV RNA and proteins are mainly found late, concomitantly with the development of AIDS and encephalitis, and the productive infection of the CNS likely occurs via blood-derived monocytes that become perivascular macrophages (Gartner, 2000; Fischer-Smith *et al*, 2001; Williams *et al*, 2001). Because a major infected cell type within the brain is the perivascular macrophages (Williams *et al*, 2001), the angiocentric distribution of lymphocytes in lesions with viral antigens, or outside of lesions next to virus-infected cells, suggests antigen-specific traffic and retention of $CD8^+$ T lymphocytes in the CNS. In our preliminary studies using SIV-gag p11c tetramers, we found a significant number of SIV-gag–specific $CD8^+$ T lymphocytes in the CNS of animals with AIDS and SIVE (unpublished data).

Our results that the majority of $CD8^+$ T lymphocytes in the CNS are also CD3-positive cells differ from a report by Mankowski *et al* (2002a). Using pig-tailed macaques coinfected with a highly pathogenic SIV clone and an immunosuppressive

SIV swarm, they found both $CD8^+$ T lymphocytes (assessed by $CD3^+/TIA\text{-}1^+$) and NK cells ($CD3^-/TIA\text{-}1^+$) predominate in animals with SIVE. We found little-to-no accumulation of $CD4^+$ T lymphocytes early or of natural killer (NK) cells in brains of our SIV-infected animals. Whether these differences are due to species difference (pig-tailed macaques versus rhesus macaques), different virus strains used (SIV/17E-Fr plus SIV/DeltaB670 versus SIVmac251), or both is not known.

In all SIVE cases we examined, CD4 staining was found on many perivascular macrophages, MNGCs, and some process-bearing cells ($CD3^-/CD4^+$), but not on lymphoid cells ($CD3^+/CD4^-$). The majority of $CD4^+$ cells we found were CD3 negative and intimately associated with CNS vessels, consistent with these cells being perivascular macrophages or inflammatory macrophages (Vazeux *et al*, 1987; Peudenier *et al*, 1991). Our findings of CD4 reactivity on brain macrophages are consistent with previous reports (Vazeux *et al*, 1987; Porwit *et al*, 1989; Li *et al*, 1991; Peudenier *et al*, 1991). Whether CD4 antigen expression on brain macrophages is critical for HIV and SIV infection *in situ* is not resolved. In this light, it is interesting to note that polymerase chain reaction (PCR) studies of virus sequences from human and monkey brain specimens often show CD4 independent viruses (Desrosiers *et al*, 1991; Kodama *et al*, 1993; Wang *et al*, 2001).

In summary, we found little-to-no accumulation of $CD4^+$ T lymphocytes and NK cells in control and SIV-infected brains. In contrast, our study has shown that $CD8^+$ T lymphocytes accumulate in the brains of macaques with AIDS and SIVE. A majority of $CD8^+$ T cells were found in conjunction with SIV-infected cells, which are almost exclusively associated with CNS vessels. The angiocentric distribution of these $CD8^+$ T cells suggests that their accumulation is triggered by viral antigens, many of which are associated with CNS perivascular macrophages. Whether the perivascular and parenchymal T cells infiltrating into the CNS of SIV-infected monkeys are recruited and retained in an antigen-specific manner, or are oligoclonal, remains to be determined.

Materials and methods

Tissue collection and processing

Necropsy specimens of brain, lymph node, and spleen from SIV-infected rhesus macaques (*Macaca mulatta*) with AIDS and encephalitis ($n = 9$), SIV-infected animals with AIDS and without encephalitis ($n = 7$), SIV-infected animals at peak viremia ($n = 5$), and 3 normal uninfected controls were investigated in the present study. Animals were infected with SIVmac251 (20 ng of SIV p27) by intravenous injection. When the animals developed AIDS, they were anesthetized with ketamine-HCl, killed by intravenous pentobarbital overdose, and exsanguinated. CNS tissues were collected in 10% neutral-buffered formalin, embedded in paraffin, and cut into 5-micron-thick sections. Adjacent tissues were snap-frozen in optimum cutting temperature compound (Miles Scientific, Elkhart, IN) by immersion in 2-methylbutane in dry ice.

Immunohistochemistry for CD4 and CD8

After incubation in an oven for 2 h at 60°C, paraffin sections were immersed in a peroxidase blocking reagent (DAKO, Carpinteria, CA) for 5 min to block endogenous peroxidase activity. Sections were treated in an electric pressure cooker for 15 min in Trilogy solution (Cell Marque, Hot Springs, AR) for deparaffinization, dehydration, and antigen retrieval, and then incubated for 10 min with a protein block (DAKO) to reduce background staining. Sections were incubated for 2 h either with a mouse primary antibody against CD4 (1:20 dilution, clone 1F6; NeoMarkers, Fremont, CA) or CD8 (1:50 dilution; clone 1A5; Novocastra, Newcastle-upon-Tyne, UK). CD4 and CD8 antigens were detected using an EnVision horseradish peroxidase–labeled polymer conjugated with secondary mouse and rabbit antibodies (DAKO). Sections were rinsed twice in 1× Tris-buffered saline (TBS) containing 0.05% Tween-20 between each reagent. The color reaction product was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAKO) as the chromogenic substrate for horseradish peroxidase. The sections were counterstained with hematoxylin, and then dehydrated and mounted. Routine inclusion of the isotype-matched negative control immunoglobulin (IgG_1; DAKO) yielded no positive staining.

Multilabel immunohistochemistry with CD3 and Glut-1

In order to investigate the distribution and immunophentype of T lymphocytes in the CNS, CD4 or CD8 staining was often followed by staining with anti–Glut-1 or anti-CD3 antibody or both. The double- or triple-label immunohistochemistry was performed with an EnVison Doublestain System (DAKO). After washing sections in water, CD4- or CD8-stained sections were incubated for 3 min with a Doublestain block (DAKO). Sections were incubated for 30 min with rabbit anti–Glut-1 (1:5000 dilution; Chemicon, Temecula, CA) or rabbit anti-human CD3 polyclonal antibody (1:350 dilution; DAKO). Detection of Glut-1 and CD3 was done using an EnVision alkaline phosphatase–labeled polymer conjugated with secondary mouse and rabbit antibodies (DAKO). The color reaction product was developed using Fast Red (DAKO) or Vector Blue (Vector Laboratories, Burlingame, CA) for Glut-1 and CD3 staining, respectively. The sections were mounted using Faramout aqueous mounting medium (DAKO). Routine inclusion of rabbit Ig fraction (DAKO) used as a negative control yielded no positive staining. For

a triple-label immunohistochemistry of CD8, CD3, and Glut-1, double-stained sections with CD8 and CD3 were subject to Glut-1 staining, as described above.

In situ *hybridization for SIV RNA*

In situ hybridization for SIV RNA was performed with digoxigenin-labeled riboprobes from Lofstrand Labs (Gaithersburg, MD; with permission from Dr. V. Hirsch and C. Brown, National Institute of Health, Rockville, MD). The probes were a pool of RNAs complementary to sequences in full-length SIVmac239 genomic RNA (Hirsch *et al*, 1997).

In situ hybridization was performed with a modification of previously published procedures under RNase-free conditions (Hu *et al*, 1998). For this, 5-micron sections of formalin-fixed, paraffin-embedded tissues were dried at 60°C for 1 h, deparaffinized in xylene twice for 5 min each, and then rehydrated in a graded ethanol series. After hydration with phosphate-buffered saline (PBS), the tissue sections were heated in a microwave oven at 800 W for 20 min with antigen unmasking solution (Vector Laboratories), and cooled for 20 min at room temperature. Sections were washed with PBS for 5 min, and immersed in 1 PBS containing 0.15% Triton X-100 for 10 min at room temperature. Sections were rinsed twice for 10 min each in 2× standard saline citrate (SSC). Before hybridization, 120 μl of prehybridization buffer containing 10% dextran sulfate, 4× SSC, 2 mM EDTA, 50% deionized formamide, 1× Denhardt's solution, 500 μg/ml herring sperm DNA, and 500 μg/ml yeast tRNA (all reagents purchased from Sigma, St. Louis, MO) was applied to sections. After incubation for 1 h at 45°C, hybridization mixtures containing SIV riboprobes (80 ng/ml in prehybridization buffer) were applied to the sections, and hybridized at 45°C overnight. The sections were subjected to two washes for 15 min at 45°C in 2 × SSC, 1 × SSC, and 0.1 × SSC, and then washed in Buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.6) for 5 min. After preincubation of sections for 30 min at room temperature with Buffer 1 containing 1% blocking reagent (Roche, Indianapolis, IN), the sections were incubated either with alkaline phosphatase–conjugated anti-digoxigenin Fab fragment (1:500 diluted in 1% blocking solution) for SIV riboprobes for 2 h at room temperature. The sections were washed in Buffer 1 twice for 10 min each, and rinsed in Buffer 2 (100 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) for 5 min. Detection of hybridized probes was done using 5-bromo-4-chloro-indolylphosphate/nitroblue tetrazolium (NBT/BCIP; Roche) as a chromogenic substrate. Sections were rinsed with water, dehydrated in a graded ethanol series, cleared in Histo-Clear (National Diagnostics, Atlanta, GA), and coverslipped with vectamount (Vector Laboratories). Negative controls included serial sections hybridized with sense probes and sections from noninfected monkey tissues.

Combined in situ *hybridization and immunohistochemistry*

For simultaneous detection of virus nucleic acid and $CD8^+$ T cells in the same section, *in situ* hybridization for SIV RNA was followed by immunohistochemistry for $CD8^+$ T cells. After incubation in an oven for 2 h at 60°C, tissue sections were deparaffinized and rehydrated in an electric pressure cooker in Trilogy solution (Cell Marque) for 15 min. The sections were then subjected to microwave pretreatment with antigen unmasking solution (Vector Laboratories). The combination of the two heat-induced epitope-retrieval methods using both pressure cooker and microwave treatment was used to achieve CD8 antigen retrieval from paraffin-embedded tissues and to improve *in situ* hybridization of viral nucleic acid. After microwave pretreatment, endogenous peroxidase activity was blocked by a 5-min incubation with 3% H_2O_2. The procedure then followed the protocol above as described for the *in situ* hybridization procedure. After *in situ* hybridization, sections were washed in 1× TBS containing 0.05% Tween-20 for 10 min. Immunohistochemistry for CD8 was performed as described above.

Quantitative evaluation of labeled T lymphocytes

Parenchymal and perivascular $CD8^+$ and $CD3^+$ cells in the cortical areas of brains were counted on at least three sections and the sum was divided by the entire section area, using National Institutes of Health software Image J. Data are expressed as the number of $CD8^+$ cells per 50 mm^2. T lymphocytes in the choroid plexus, the meninges, and the lumen of blood vessels were excluded because areas of these compartments varied from case to case. The $CD8^+/CD3^+$ ratios were calculated in a single section of the cortex in every case with double immunostaining. The distribution of $CD8^+$ T cells in association with vessels and infected cells was expressed as mean percentages of all $CD8^+$ T cells using two to three sections for each case. All statistical analyses were performed using the JMP (SAS statistical package) software.

References

Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schröder R, Deckert M, Schmidt S, Ravid R, Rajewsky K (2000). Clonal expansions of $CD8^+$ T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* **192:** 393–404.

Bauer J, Rauschka H, Lassmann H (2001). Inflammation in the nervous system: the human perspective. *Glia* **36:** 235–243.

Bell JE, Busuttil A, Ironside JW, Rebus S, Donaldson YK, Simmonds P, Peutherer JF (1993). Human immunodeficiency virus and the brain: investigation of virus load and neuropathologic changes in pre-AIDS subjects. *J Infect Dis* **168:** 818–824.

Bien CG, Bauer J, Deckwerth TL, Wiendl H, Deckert M, Wiestler OD, Schramm J, Elger CE, Lassmann H (2002). Destruction of neurons by cytotoxic T cells: a new pathogenic mechanism in Rasmussen's encephalitis. *Ann Neurol* **51:** 311–318.

Boche D, Khatissian E, Gray F, Falanga P, Montagnier L, Hurtrel B (1999). Viral load and neuropathology in the SIV model. *J NeuroVirol* **5:** 232–240.

Broadwell RD, Charlton HM, Ebert P, Hickey WF, Villegas JC, Wolf AL (1990). Angiogenesis and the blood-brain barrier in solid and dissociated cell grafts within the CNS. *Prog Brain Res* **82:** 95–101.

Chakrabarti L, Hurtrel M, Maire MA, 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.

Desrosiers RC, Hansen-Moosa A, Mori K, Bouvier DP, King NW, Daniel MD, 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.

Fischer-Smith T, Croul S, Sverstiuk AE, Capini C, L'Heureux D, Regulier EG, Richardson MW, Amini S, Morgello S, Khalili K, Rappaport J (2001). CNS invasion by CD14+/CD16+ peripheral blood-derived monocytes in HIV dementia: perivascular accumulation and reservoir of HIV infection. *J NeuroVirol* **7:** 528–541.

Gartner S (2000). HIV infection and dementia. *Science* **287:** 602–604.

Griffin DE, Levine B, Tyor WR, Irani DN (1992). The immune response in viral encephalitis. *Sem Immunol* **4:** 111–119.

Hickey WF (1991). Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathol* **1:** 97–105.

Hirsch VM, 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.

Hu J, Pope M, Brown C, O'Doherty U, Miller CJ (1998). Immunophenotypic characterization of simian immunodeficiency virus-infected dendritic cells in cervix, vagina, and draining lymph nodes of rhesus monkeys. *Lab Invest* **78:** 435–451.

Hurtrel B, Chakrabarti L, Hurtrel M, Maire MA, Dormont D, Montagnier L (1991). Early SIV encephalopathy. *J Med Primatol* **20:** 159–166.

Iwasaki Y, Sako K, Tsunoda I, Ohara Y (1993). Phenotypes of mononuclear cell infiltrates in human central nervous system. *Acta Neuropathol (Berl)* **85:** 653–657.

Jassoy C, Johnson RP, Navia BA, Worth J, Walker BD (1992). Detection of a vigorous HIV-1-specific cytotoxic T lymphocyte response in cerebrospinal fluid from infected persons with AIDS dementia complex. *J Immunol* **149:** 3113–3119.

Katsetos CD, Fincke JE, Legido A, Lischner HW, de Chadarevian JP, Kaye EM, Platsoucas CD, Oleszak EL (1999). Angiocentric $CD3^+$ T-cell infiltrates in human immunodeficiency virus type 1-associated central nervous system disease in children. *Clin Diagn Lab Immunol* **6:** 105–114.

Kaul M, Garden GA, Lipton SA (2001). Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* **410:** 988–994.

Kodama T, Mori K, Kawahara T, Ringler DJ, Desrosiers RC (1993). Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with AIDS. *J Virol* **67:** 6522–6534.

Lackner AA (1994). Pathology of simian immunodeficiency virus induced disease. *Curr Top Microbiol Immunol* **188:** 35–64.

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.

Lane JH, Sasseville VG, Smith MO, Vogel P, Pauley DR, Heyes MP, Lackner AA (1996). Neuroinvasion by simian immunodeficiency virus coincides with increased numbers of perivascular macrophages/microglia and intrathecal immune activation. *J NeuroVirol* **2:** 423–432.

Lellouch-Tubiana A, Fohlen M, Robain O, Rozenberg F (2000). Immunocytochemical characterization of long-term persistent immune activation in human brain after herpes simplex encephalitis. *Neuropathol Appl Neurobiol* **26:** 285–294.

Li SL, Kaaya EE, Feichtinger H, Putkonen P, Parravicini C, Bottiger D, Biberfeld G, Biberfeld P (1991). Monocyte/macrophage giant cell disease in SIV-infected cynomolgus monkeys. *Res Virol* **142:** 173–182.

Mankowski JL, Clements JE, Zink MC (2002a). Searching for clues: tracking the pathogenesis of human immunodeficiency virus central nervous system disease by use of an accelerated, consistent simian immunodeficiency virus macaque model. *J Infect Dis* **186(Suppl 2):** S199–S208.

Mankowski JL, Queen SE, Tarwater PM, Fox KJ, Perry VH (2002b). Accumulation of beta-amyloid precursor protein in axons correlates with CNS expression of SIV gp41. *J Neuropathol Exp Neurol* **61:** 85–90.

Marcondes MC, Phillipson CA, Fox HS (2003). Distinct clonal repertoire of brain CD8+ cells in simian immunodeficiency virus infection. *AIDS* **17:** 1605–1611.

Mason DY, Cordell JL, Gaulard P, Tse AGD, Brown MH (1992). Immunohistological detection of human cytotoxic/suppressor T cells using antibodies to a CD8 peptide sequence. *J Clin Pathol* **45:** 1084–1088.

Miller RF, Isaacson PG, Hall-Craggs M, Lucas S, Gray F, Scaravilli F, An SF (2004). Cerebral CD8+ lymphocytosis in HIV-1 infected patients with immune restoration induced by HAART. *Acta Neuropathol (Berl)* **108:** 17–23.

Parravicini CL, Petrén AL, Vago L, Costanzi G, Gluckman JC, Gallo RC, Biberfeld P (1989). HIV encephalopathy and lymphadenopathy: cells associated with viral antigens. *APMIS Suppl* **8:** 33–39.

Petito CK, Adkins B, McCarthy M, Roberts B, Khamis I (2003). CD4+ and CD8+ cells accumulate in the brains of acquired immunodeficiency syndrome patients with human immunodeficiency virus encephalitis. *J NeuroVirol* **9:** 36–44.

Peudenier S, Hery C, Ng KH, Tardieu M (1991). HIV receptors within the brain: a study of CD4 and MHC-II on human neurons, astrocytes and microglial cells. *Res Virol* **142:** 145–149.

Porwit A, Parravicini C, Petren AL, Barkhem T, Costanzi G, Josephs S, Biberfeld P (1989). Cell association of HIV in AIDS-related encephalopathy and dementia. *APMIS* **97:** 79–90.

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

Reinhart TA, Rogan MJ, Huddleston D, Rausch DM, Eiden LE, Haase AT (1997). Simian immunodeficiency virus burden in tissues and cellular compartments during clinical latency and AIDS. *J Infect Dis* **176:** 1198–1208.

Sethi KK, Naher H, Stroehmann I (1988). Phenotypic heterogeneity of cerebrospinal fluid-derived HIV-specific and HLA-restricted cytotoxic T-cell clones. *Nature* **335:** 178–181.

Sopper S, Sauer U, Hemm S, Demuth M, Muller J, Stahl-Hennig C, Hunsmann G, ter Meulen V, Dorries R (1998). Protective role of the virus-specific immune response for development of severe neurologic signs in simian immunodeficiency virus-infected macaques. *J Virol* **72:** 9940–9947.

Streilein JW (1995). Unraveling immune privilege. *Science* **270:** 1158–1159.

Togo T, Akiyama H, Iseki E, Kondo H, Ikeda K, Kato M, Oda T, Tsuchiya K, Kosaka K (2002). Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. *J Neuroimmunol* **124:** 83–92.

Tyor WR, Glass JD, Griffin JW, Becker PS, McArthur JC, Bezman L, Griffin DE (1992). Cytokine expression in the brain during the acquired immunodeficiency syndrome. *Ann Neurol* **31:** 349–360.

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.

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.

Wang TH, Donaldson YK, Brettle RP, Bell JE, Simmonds P (2001). Identification of shared populations of human immunodeficiency virus type 1 infecting microglia and tissue macrophages outside the central nervous system. *J Virol* **75:** 11686–11699.

Weidenheim KM, Epshteyn I, Lyman WD (1993). Immunocytochemical identification of T-cells in HIV-1 encephalitis: implications for pathogenesis of CNS disease. *Mod Pathol* **6:** 167–174.

Williams K, Hickey WF (1995). Traffic of lymphocytes into the CNS during inflammation and HIV infection. *J Neuro-Aids* **1:** 31–55.

Williams KC, Corey S, Westmoreland SV, Pauley D, Knight H, deBakker C, Alvarez X, Lackner AA (2001). Perivascular macrophages are the primary cell type productively infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS. *J Exp Med* **193:** 905–915.

Williamson SLH, Steward M, Milton I, Parr A, Piggott NH, Krajewski AS, Angus B, Horne CHW (1998). New monoclonal antibodies to the T cell antigens CD4 and CD8. Production and characterization in formalin-fixed paraffin-embedded tissue. *Am J Pathol* **152:** 1421–1426.