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CD4+ and CD8+ cells accumulate in the brains of acquired immunodeficiency syndrome patients with human immunodeficiency virus encephalitis

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To test the hypothesis that CD4+ T lymphocytes accumulate in brains of end-stage acquired immunodeficiency syndrome (AIDS) patients, we examined T-lymphocyte subsets in the CA1, CA3, and CA4 regions of the hippocampus of AIDS patients with ($n = 10$) and without ($n = 11$) human immunodeficiency virus encephalitis (HIVE) plus controls ($n = 7$). HIV p24 antigen was common in monocytic cells and rare in activated/memory CD45RO+ lymphocytes. Hippocampal activated/memory CD45RO+ T lymphocytes significantly increased ($P < .001$) in seven of the eight hippocampal subregions with hippocampal HIVE (1.14 ○ 1.4 T cells/high-power field [hpf]), but AIDS hippocampus without HIVE were similar to controls (0.03 ○ 0.07 T cells/hpf and 0.03 ○ 0.09 T cells/hpf, respectively). CD45RO+ and CD3+ lymphocytes were similar in numbers and distribution, whereas CD4+ and CD8+ lymphocytes were weakly immunoreactive and less frequent. All four lymphocyte subtypes were present in perivascular spaces and microglial nodules of HIVE, and had direct contact with neurons. Monocytes, microglia, and multinucleated giant cells were immunoreactive for CD4 in AIDS cases with hippocampal HIVE but microglia in remaining AIDS cases and controls were CD4○ . CD68+ macrophages significantly increased in hippocampus of HIVE patients ($P < .05$) and were predominately perivascular in the absence of local HIVE. These studies show that CD4+ T lymphocytes, as well as CD8+ T lymphocytes, participate in the local inflammatory response of HIVE in end-stage AIDS patients, and suggest that their recruitment requires local HIV infection. The perineuronal location of CD4+ cells provides the potential for lymphocyte-mediated neuronal injury or trans-receptor–mediated neuronal infection. *Journal of NeuroVirology* (2003) **9,** 36–44.

Keywords: AIDS; CD4+ T lymphocytes; HIV encephalitis; inflammation; neurons

Introduction

The central nervous system (CNS) is an important site of human immunodeficiency virus (HIV)-associated injury. Its vulnerability was first highlighted by Snider *et al* (1983) who described a 30% incidence of CNS disease in acquired immunodeficiency syndrome (AIDS) patients. These included not only infections and lymphomas consequent to AIDS-related immunosuppression, but also a newly recognized dementing illness termed AIDS dementia complex or HIV-associated cognitive/motor dysfunction (HAD) (Navia *et al*, 1986; Janssen *et al*, 1991). Neuronal injury and HAD are closely related to brain infection by HIV, although productive HIV infection is limited to cells of monocyte lineage. In patients with encephalitis due to HIV (HIVE), the characteristic inflammatory microglial nodules (MGNs) and infected

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monocyte/macrophage cells are most numerous in subcortical nuclei and cerebral white matter, although the MGNs can occur throughout the brain and spinal cord. Suggested mechanisms for neuronal injury include cytotoxic substances released from activated or infected monocytic cells, exposure to neurotoxic viral proteins, or damage mediated by astrocyte dysfunction secondary to restricted HIV infection of these cells (Gendelman *et al*, 1994; Nath and Geiger, 1998).

Direct neuronal infection by HIV is an alternative explanation for neuronal injury and death. Hippocampal neurons, isolated from AIDS brains by laser capture microdissection, contain HIV *nef* and, less frequently, *env* gene sequences (Torres-Muñoz *et al*, 2001). Two other studies detected HIV *gag* sequences in brain neurons using combined gene amplification and *in situ* hybridization (Nuovo *et al*, 1994; Bagasra *et al*, 1996), although the results with these *in situ* techniques are controversial and not supported by subsequent investigations using similar techniques (Takahashi *et al*, 1996: Sharer *et al*, 1996; An *et al*, 1999). In culture, neurons and astrocytes are more susceptible to injury and infection by T lymphocyte–tropic virus and virally derived gp 120 protein than they are to macrophage-tropic strains (Nath *et al*, 1995; Hesselgesser *et al*, 1998; McCarthy *et al*, 1998; Zheng *et al*, 1999; O'Hagen *et al*, 1999; Klein *et al*, 1999). This selective neuronal vulnerability is related to their constitutively high expression levels of the T-tropic virus–using CXCR4 HIV chemokine coreceptors (Lavi *et al*, 1997). These neuronal coreceptors increase with AIDS and with HIVE (van der Meer *et al*, 2000; Petito *et al*, 2001), in association with a concomitant decrease in neuronal M-tropic virus–using CCR5 chemokine coreceptors (Petito *et al*, 2001).

In the present study, we tested the hypothesis that CD4+ T lymphocytes accumulate in brains of end-stage AIDS patients. If present, these lymphocytes could participate in brain injury by activating latent virus in neuroectodermal cells (Tornatore *et al*, 1991), by permitting trans-receptor viral entry into CD4-negative neuroectodermal cells (Speck *et al*, 1999), or by up-regulating neuronal expression of HIV chemokine coreceptors (Speth *et al*, 2000). Although peripheral CD4+ T-lymphocyte counts are low in end-stage AIDS patients, they represent the principal source of systemic HIV-1 infection at this time (van der Ende *et al*, 1999).

We used paraffin-embedded autopsy brain sections to determine the numbers and distribution of inflammatory cells in hippocampus of AIDS patients with HIVE, AIDS patients without HIVE (HIVnE), and controls with normal brains and no evidence of HIV infection. Immunohistochemistry identified T-lymphocyte subsets, macrophages, and HIV-1 protein. We described the results according to patient group and according to hippocampal subregions because our prior studies suggest the CA3 and CA4 are selectively vulnerable to HIV-associated injury (Petito *et al*, 2001). A preliminary report has been published (Petito *et al*, 2000).

Results

Paraffin-embedded blocks of hippocampus were studied in 10 AIDS patients with HIVE (HIVE), 11 AIDS patients without HIVE (HIVnE), and 7 control patients. The clinical characteristics and neuropathology of these patients have been described previously; case no. 3 from that prior study was unavailable for the present one (Petito *et al*, 2001). As reviewed in our earlier paper, risk factor information was absent in most cases. Four of the 10 HIVE patients contained microglial nodules of HIVE in the hippocampal pyramidal cell layer, which were present in a total of 8 of their 12 hippocampal subregions. Opportunistic infection and lymphoma (OI/L) were absent in hippocampal sections but present in remote brain regions in four HIVE patients and none of the HIVnE patients. One control hippocampus contained a micrometastasis from a systemic adenocarcinoma and one had changes suggestive of hyperacute hypoxia-ischemia. AIDS patients were younger than controls by approximately a decade; contained a higher percentage of men; and had similar, or longer, postmortem intervals (Petito *et al*, 2001).

In our positive tissue controls, as well as the three patient groups, immunostaining for CD45RO and CD3 immunoreactivity was more intense in all samples when compared with that for CD4 and CD8, despite the use of EDTA buffer and brief or no exposure to hydrogen peroxide quenching as per manufacturer's directions for CD4 and CD8. As a result, we quantitated the numbers of CD45RO+ cells but not the numbers of CD4+ and CD8+ cells.

Perivascular and parenchymal CD45RO+ and CD3+ T lymphocytes and CD68+ macrophages were rare or absent in controls (Table 1). Microglia were CD4≈. Because controls were older than the AIDS cases, we also examined another seven control brains from patients between 25 and 48 years of age (mean age of 35.4 ≈ 8 years); none contained lymphocytes in the hippocampus.

Perivascular T lymphocytes were increased in perihippocampal white matter in the four AIDS cases with hippocampal HIVE, but were rare or absent in the pyramidal cell layer of all patient groups and perihippocampal white matter of controls and AIDS cases without hippocampal HIVE. The perivascular cells included CD45RO+ activated/memory T lymphocytes (Figure 1**A**) and CD3+, CD4+, and CD8+ T lymphocytes (Figure 1**C–E**). We found no evidence that the CD45RO+ lymphocytes were immunoreactive for HIV p24 antigen on serial sections (Figure 1**B**).

Parenchymal CD45RO+ and CD3+ T lymphocytes were abundant in seven of the eight hippocampal

Table 1 Numbers of T lymphocytes and macrophages in hippocampus

	CD45RO+ T lymphocytes			*CD68+ macrophages*		
Patient groups	*CA1*	*CA3*	*CA4*	*CA1*	*CA3*	*CA4*
HIVE ($n = 8$)	$0.35 \approx 0.24$	$0.35 \approx 0.21$	$0.68 \approx 1.40$	$1.00 \approx 0.4^{\approx}$	$0.69 \approx 0.34^{\approx}$	$0.69 \approx 0.17$
HIVnE ($n = 8$)	$0.01 \approx 0.00$	$0.01 \approx 0.19$	$0.02 \approx 0.38$	$0.15 \approx 0.35$	$0.09 \approx 0.32$	$0.16 \approx .017$
Control ($n = 5$)	$0.06 \approx 0.03$	$0.11 \approx 0.05$	$0.02 \approx 0.05$	$0.38 \approx 0.44$	$0.39 \approx 0.41$	$0.07 \approx 0.22$

Note. Data are mean $\approx$ *SE* $^{\approx}P < .05$ when compared to control and HIVnE (Kruskal-Wallis and ANOVA). *Abbreviations*: *n*: number of cases; CD45RO+ T lymphocytes: average number $\approx$ *SE* per high power field; CD68+ macrophages: average number $\approx$ *SE* of CD68+ monocytes/macrophages per high power field; HIVE: AIDS patients with HIV encephalitis; HIVnE: AIDS patients without HIVE; CA1, CA3, CA4: anatomical areas of pyramidal neurons of the hippocampus.

subregions with local HIVE, but were rare or absent in the hippocampus of the HIVnE patient group and in the HIVE patient group without hippocampal HIVE (Table 1). Parenchymal CD4+ and CD8+ T lymphocytes also were encountered; as discussed above, their immunoreactivity was less than that of CD45RO and CD3. All four lymphocyte subsets (CD3+, CD45RO+, CD4+, and CD8+) were found in the microglial nodules of HIVE. Additionally, CD45RO+ T lymphocytes were in direct contact with pyramidal cell neurons in the four AIDS cases with hippocampal HIVE (Figure 2**A**, **D**). Such T lymphocyte–neuronal contact was found only once in one of the remaining 6 HIVE patients without hippocampal HIVE and was absent in the 11 HIVnE AIDS patient group and in the 7 controls. Some of the lymphocyte-neuronal contacts were in the regions of the MGNs whereas others were removed from the immediate vicinity of the HIVE microglial nodules as well as the perivascular infiltrates, suggesting migration of the T cells from one of these regions. Perineuronal T lymphocytes included CD4+ and CD8+ cells (Figure 2**B**, **C**). Rare parenchymal CD45RO+ T lymphocytes were immunoreactive for HIV p24 antigen on serial sections (Figure 2**D**, **E**).

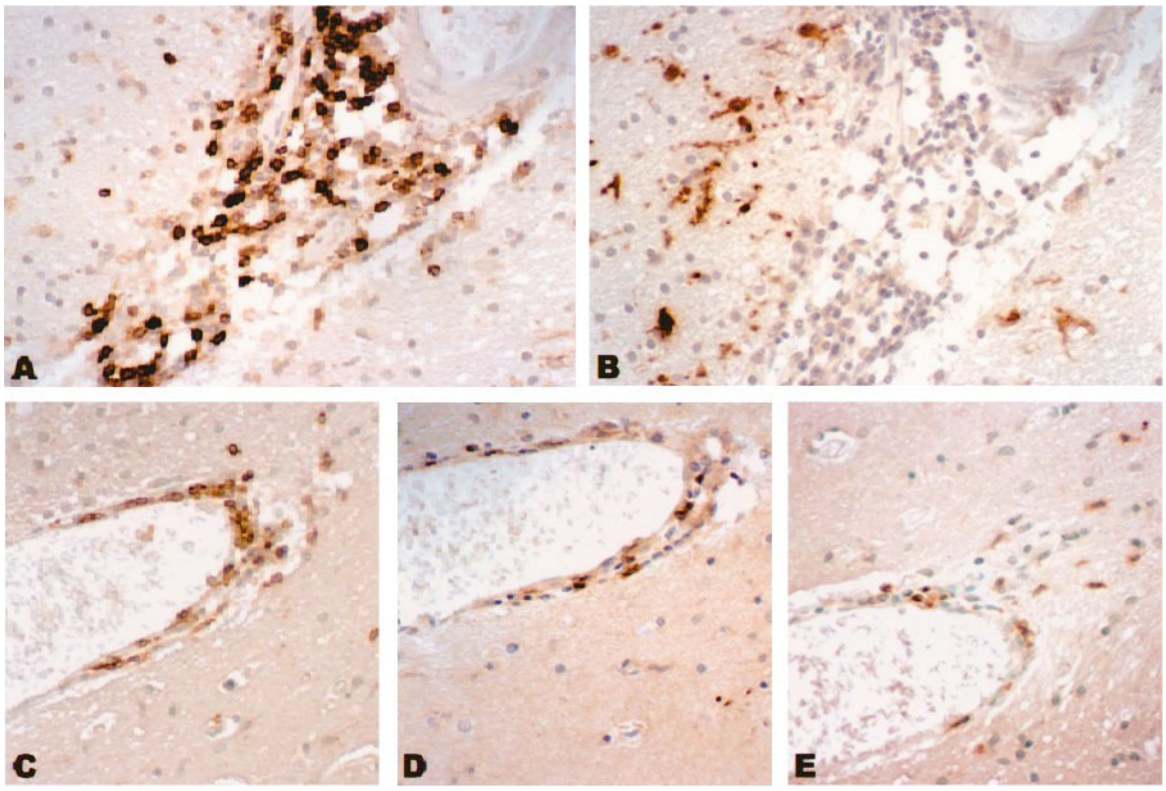

Figure 1 (**A**, **B**) AIDS patient with hippocampal HIVE. Dense perivascular lymphocyte infiltrate in perihippocampal white matter contains numerous activated/memory CD45RO+ T lymphocytes (**A**). HIV p24 immunoreactivity (**B**) is detected in perivascular macrophages and in adjacent brain monocytes and microglia but not in perivascular lymphocytes. (**C**, **D**, **E**) AIDS patient with hippocampal HIVE. Perivascular T-cell infiltrates include CD3+ (**C**), CD4+ (**D**), and CD8+ (**E**) lymphocytes. Parenchymal infiltration of these T cells also is seen. Hematoxylin counterstain. Original magnification, ≈400.

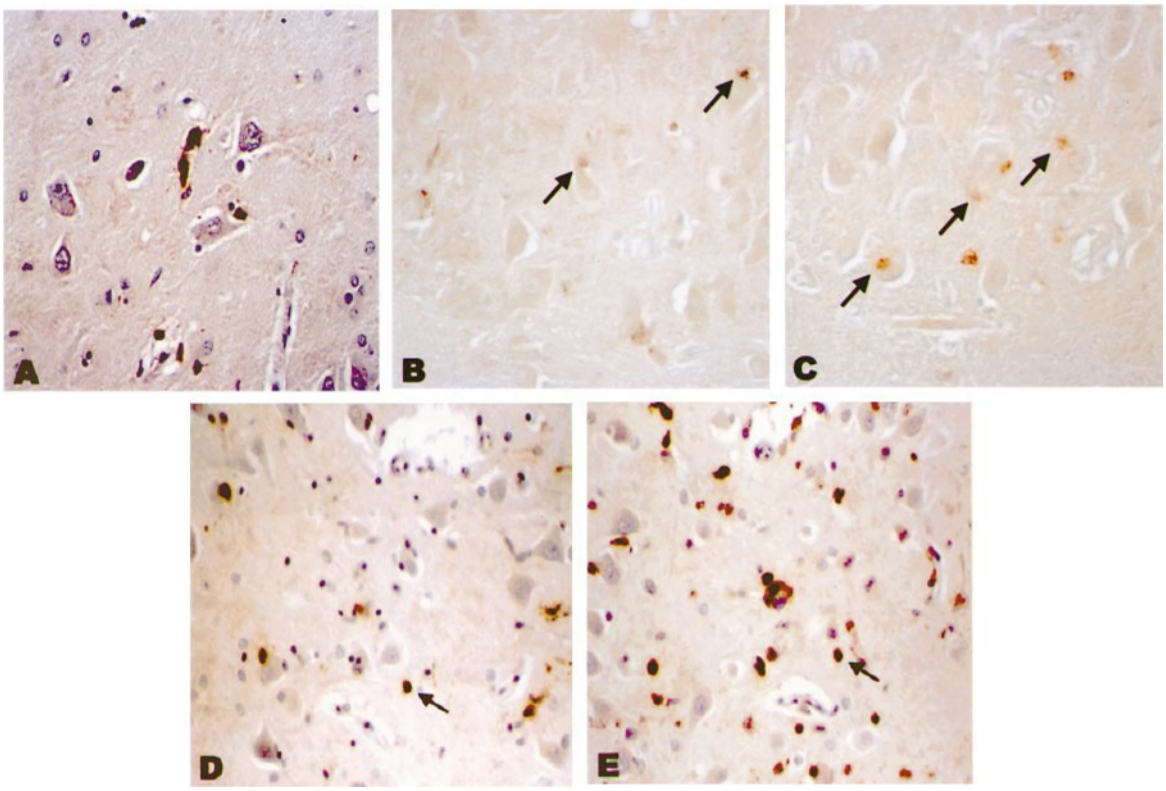

Figure 2 (**A**, **B**, **C**) AIDS with hippocampal HIVE; CA4 region (**A**) and serial sections through the CA3 region of a second case (**B** and **C**). Direct cell-to-cell contact between pyramidal neurons and lymphocytes includes CD45RO+ T lymphocytes (**A**) and CD4+ T lymphocytes (**B**), and CD8+ lymphocytes (**C**); (arrows). (**D**, **E**) AIDS patient with hippocampal HIVE inflammation, CA4 region. Inflammatory response contains CD45RO+ T lymphocytes (**D**, *arrow*), one of which also is immunoreactive for HIV p24 on its serial section (**E**, *arrow*). In **D**, two perineuronal CD45RO+ T cells left are in direct contact with neuronal cell bodies (*left*). Hematoxylin counterstain (**A**, **D–E**). Original magnification, ≈400.

Monocytes, microglia, and multinucleated giant cells also were CD4+ and occasionally directly abutted neuronal cytoplasm in those AIDS cases with hippocampal HIVE (data not shown).

We quantitated the numbers of hippocampal CD45RO+ T lymphocytes and CD68+ macrophages. We used the CD45RO immunostain rather than the CD4 and CD8 immunostains because immunoreactivity for the latter two was not strong. We detected a higher mean number of hippocampal CD45RO+ T lymphocytes in the HIVE patient group ($n = 10$) than in the HIVnE patient group ($n = 11$) and the control patients ($n = 7$), but significant differences were absent when expressed according to patient groups (Table 1). However, when we analyzed the results according to the presence or absence of local hippocampal HIVE in the AIDS patients and controls (Figure 3), we found a highly significant increase in T lymphocytes in those hippocampal regions with HIVE inflammatory nodules ($P < .001$). The mean number of CD45RO+ T lymphocytes high-power field (hpf) ≈ *SE* was 1.14 ≈ 1.1 in the 8 HIVE-containing hippocampal subregions of 4 AIDS patients versus 0.03 ≈ 0.03 cells/hpf in 55 HIVE-negative hippocampal subregions of the remaining 17 AIDS patients (6 HIVE patients without local hippocampal plus 11 HIVnE

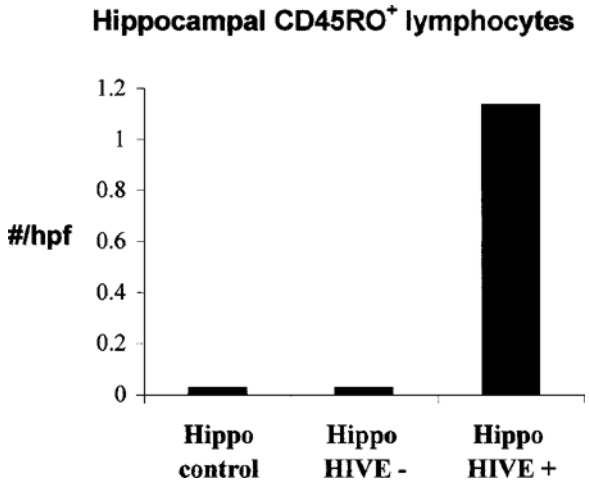

Figure 3 Bar graph showing the number of CD45RO+ T lymphocytes per high power field (#/hpf) in 17 hippocampal subregions of 7 control cases (Hippo control), in 55 normal hippocampal regions (Hippo HIVE≈) of 17 AIDS patients, and 8 hippocampal regions with local HIVE from 4 AIDS cases (Hippo HIVE+) in 4 AIDS patients. Hippo control: 0.03 ≈ 0.67 cells/hpf; Hippo HIVE≈: 0.03 ≈ 0.03 cells/hpf; Hippo HIVE+: 1.14 ≈ 1.1 cells/hpf. HIVE+ significantly differs from controls and HIVE≈ groups ($P < .001$; Kruskal-Wallis and ANOVA).

patients) and 0.03 ≈ 0.67 cells/hpf in 17 hippocampal subregions of the 7 controls. There was no apparent correlation between the number of hippocampal T cells and OI/L elsewhere in the brain. CD68+ macrophages significantly increased in all three hippocampal regions of the HIVE patient but not in the HIVnE patient group (Table 1).

Discussion

In the present study, we found a close relationship between perivascular and parenchymal hippocampal T lymphocytes and local HIVE inflammatory infiltrates. In the absence of local HIVE, the numbers of perivascular and parenchymal T lymphocytes in the hippocampus of AIDS patients were similar to that of controls. Not surprisingly, activated/memory T cells were in abundance, reflecting the fact that activated, rather than naive, T cells pass through the blood-brain barrier, as discussed below. In the AIDS brains, they intermingled with the other inflammatory components of the microglial nodules, including activated microglial cells, monocytes, and multinucleated giant cells. In addition, T lymphocytes directly abutted neuronal cytoplasm in all four cases with hippocampal HIVE. The presence of rare HIV-1–infected T lymphocytes suggests that T-tropic virus may be present in brains of AIDS patients. This viral strain is more neurotropic than M-tropic variants and thus could more readily damage neuroectodermal cells. However, their numbers were low and thus the significance of HIV-infected brain lymphocytes is unclear.

Prior studies that examined the relationship between AIDS and brain lymphocyte infiltrate have not yielded uniform results. Tyor *et al* (1992) and Wesselingh *et al* (1993) found that the numbers of perivascular T lymphocytes, and the amount of interferon-gamma mRNA, in frontal cortex and white matter were similar in AIDS versus control brains. Other studies (Bell *et al*, 1993; Weidenheim *et al*, 1993; Falangola *et al*, 1995; Katsetos *et al*, 1999), however, demonstrated AIDS-related T-lymphocyte infiltrates in leptomeninges, perivascular, and intramural compartments of parenchymal blood vessels and choroid plexus. In children, the perivascular infiltrates were intense. Either CD45RO or CD3 monoclonal antibodies (mAbs) were used in these studies; none examined the CD4 versus CD8 subtypes. The apparent discrepancies between distribution of vascular T cells in AIDS brains in the above studies could be related to antibody source or tissue preparation; alternatively, the intensity of lymphocyte inflammation may be related to such patient demographics as the incidence of drug abuse, which is known to increase nonspecific inflammation in brain.

When activated, T lymphocytes normally enter the CNS in low numbers (Wekerle *et al*, 1986; Hickey *et al*, 1991) where they concentrate in the meninges, ventricular spaces, and perivascular spaces. Their adhesion and migration are regulated by intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), E-selectin, and vascular cell adhesion molecule-1 (Wong *et al*, 1999). Their low numbers in normal brain may be due to restricted entry at the level of the blood-brain barrier (Wisniewski and Lossinsky, 1991), restricted production of chemoattractant chemokines, or induction of T-cell apoptosis (see Wisniewski and Lossinsky, 1991; Bauer *et al*, 1998; Carson *et al*, 1999). Perineural lymphatics allow drainage of CNS lymphocytes and proteins to the upper cervical lymph nodes (Cserr *et al*, 1992; Svenningsson *et al*, 1995; Seabrook *et al*, 1998). This pathway provides a route by which infected T cells from the CNS can reach systemic organs; in addition, it allows CNS antigens to reach the periphery from where they may elicit a peripheral immune response (Okamoto *et al*, 1999).

A number of HIV-associated changes in brain or brain endothelium may enhance migration of T cells into brains of AIDS patients. They include induced or enhanced endothelial expression of vascular adhesion molecules (Sasseville *et al*, 1992; Nottet *et al*, 1996; Seigneur *et al*, 1997) or elevation of serum tumor necrosis factor (TNF) alpha, which up-regulates or induces vascular adhesion molecules (Hurwitz *et al*, 1994). In addition, HIV infection of CD4+ lymphocytes as well as macrophages enhances their migration through *in vitro* models of the blood-brain barrier (Dhawan *et al*, 1995; Nottet *et al*, 1996; Lane *et al*, 1996). The high levels of brain endothelial CXCR4 chemokine coreceptors for HIV may allow close interaction with HIV-infected immune cells (Volin *et al*, 1998; Gupta *et al*, 1998) and thus act as vascular adhesion molecules for HIV-infected cells. Lastly, cocaine exposure, which is present in a subset of AIDS patients, enhances cerebrovascular permeability (Zhang *et al*, 1998) and increases endothelial adhesion molecule expression and lymphocyte migration (Gan *et al*, 1999).

The increases in brain T cells, as shown in the present study, could be related to the enhanced stimuli for immune cell trafficking in AIDS brains, as reviewed above. Alternatively, T-cell increases could be secondary to a reduction in their normal egress via the cerebrospinal fluid into regional cervical lymph nodes. Lastly, T cells, particularly those in or around blood vessels, may be protected against their usual removal by apoptosis. Potential inhibitory factors to T-cell apoptosis include the dysfunction of the usual antigen-presenting properties of perivascular astrocytes that prime T lymphocytes for apoptosis (Gold *et al*, 1996) or the induction or enhancement of antiapoptotic gene products in AIDS brains (Krajewski *et al*, 1997).

Brain lymphocytes may injure the CNS in AIDS patients by a number of mechanisms. First, T lymphocytes secrete bioactive substances with complex interactions that alter a wide range of cytokines

and chemokines; some, such as tumor necrosis factor alpha, are neurotoxic. Second, the direct contact between T lymphocytes and neurons raises the potential for lymphocyte-mediated neuronal toxicity. T cell–mediated cell damage usually takes place in major histocompatability complex (MHC) antigen-expressing cells via the Fas/FasL or perforin/granzyme pathways, and lymphocyte-mediated toxicity can affect MHC-negative cells as well (Russell and Ley, 2002). To date, neuronal MHC expression has not been detected in AIDS brains (Achim *et al*, 1991; Kennedy and Gairns, 1992); however, neuronal MHC expression has been found in other inflammatory conditions in association with CD8+ lymphocyte-mediated neuronal apoptosis (Bien *et al*, 2002). A third potential mechanism of T lymphocyte–mediated brain injury includes CD4+ T cells induction of sustained brain infection and inflammation via their interaction with infected neuroectodermal cells. This latter hypothesis arises from a study by Tornatore *et al* (1991) who found that exposure to T-cell factors will stimulate new production of p24 antigen by astrocytes harboring a persistent, nonproductive HIV infection.

Neuronal damage also could result from their proximity to CD4+ cells. Both the CD4+ lymphocytes and CD4+ microglia could mediate HIV entry into neurons by contributing their CD4+ receptor for viral entry into neurons, which express HIV chemokine coreceptors but not CD4 receptors. Such trans-receptor mechanisms for viral entry occur in cultured astrocytes; alone, these chemokine receptor–bearing cells are not infected by HIV unless cocultured with CD4+ cells (Speck *et al*, 1999).

Methods and materials

Patient groups
We used archival paraffin-embedded formalin-fixed blocks of hippocampus from a prior study that included AIDS patients with and without HIVE and controls with normal brains and no evidence of HIV infection (Petito *et al*, 2001). The brains had been fixed in 10% buffered formalin for 2 weeks prior to brain dissection and paraffin embedding. We made the diagnosis of HIVE based on the presence of at least one microglial nodule in one brain section that had the characteristic multinucleated giant cell of HIV infection, according to the criteria of Budka *et al* (1991).

Immunohistochemistry
We identified inflammatory cells and productive HIV infection with primary antibodies to identify the following cell-specific antigens: CD3 for a pan-T lymphocyte marker (1:600), from Santa Cruz Biotechnology (Santa Cruz, CA), CD45RO for activated/memory T lymphocytes (1:25 dilution), CD68 for monocyte/macrophages (1:400 dilution), and HIV-1 p24 protein for HIV (1:100 dilution), from Dako (Burlingame, CA). Immunohistochemistry was done for CD4 and CD8 T-lymphocyte receptors (1:500 and 1:80 dilutions, respectively), from Novacastro Lab (Newcastle-upon-Tyne, England), on those cases containing hippocampal CD45RO+ T cells. After incubation with the primary antibody, sections were sequentially incubated in biotinylated secondary antibody, avidin-biotin complex, 3,3′-diaminobenzidine, and hydrogen peroxidase. Antigen retrieval by 5′ microwaving in citrate buffer preceded immunohistochemistry for CD45RO, CD3, and p24 and EDTA buffer for CD4 and CD8; pepsin digestion preceded immunohistochemistry for CD68. On those cases with T-cell infiltrates, we performed single immunohistochemistry for CD45RO+ T lymphocytes and for HIV-1 p24 protein on serial sections of hippocampus to determine if any T lymphocytes harbored productive HIV-1 infection. We elected not to perform double-label immunohistochemistry with HIV p24 and lymphocyte markers; in our experience, their small perikarya interfered with interpretation of color differences (Falangola *et al*, 1995). Positive controls included human tonsil or lymph node, intravascular lymphocytes, and a known case of HIVE. Substitution of buffer for the mAb was used as a negative control.

Outcome measurements
Results for CD45RO+ and CD3+ T lymphocytes were similar. This is consistent with the fact that T lymphocytes in brain are of the activated phenotype (Hickey *et al*, 1991) and thus would be expected to be CD45RO+ as well as CD3+. We counted the total number of CD45RO+ T lymphocytes per 400≈ hpf in the pyramidal cell layer of the CA1, CA3, and CA4 regions of the hippocampus. The number of hpf per case averaged $41.1 \approx 12$, $39.3 \approx 13.9$, and $42.3 \approx 13.2$ for each hippocampal region, respectively. We determined differences in CD68+ monocytes/macrophages by cell numbers per hpf in the three hippocampal subregions. We expressed the results according to patient group (AIDS with HIVE, AIDS without HIVE, and controls) and according to the presence or absence of local HIVE inflammatory microglial nodules in the three hippocampal subregions in the AIDS patients, plus controls. We did not quantitate the CD4+ and CD8+ lymphocytes due to their lower immunoreactivity in the paraffin sections.

Data reduction and analysis
All slides were examined without knowledge of the patient group, although the presence of the inflammatory lesions of HIVE precluded blinded evaluation in those slides in which this was present. Because normality and equal variances between groups were absent when examined by normal probability plots and Hartley's F-Max Test, we used the Kruskal-Wallis test to determine significant differences for lymphocytes and macrophages; when P value was $<.05$, identification of group differences was determined by using least significant differences (LSD) in one-way analysis of variance (ANOVA).

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