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CNS invasion by CD14+/CD16+ peripheral blood-derived monocytes in HIV dementia: perivascular accumulation and reservoir of HIV infection

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Increases in circulating CD14+/CD16+ monocytes have been associated with HIV dementia; trafficking of these cells into the CNS has been proposed to play an important role in the pathogenesis of HIV-induced neurological disorders. This model suggests that events outside the CNS leading to monocyte activation initiate the process leading to HIV dementia. To investigate the role of this activated monocyte subset in the pathogenesis of HIV dementia, we examined brain specimens from patients with HIV encephalopathy (HIVE), HIV without encephalopathy, and seronegative controls. An accumulation of perivascular macrophages was observed in HIVE. The majority of these cells identified in microglial nodules and in the perivascular infiltrate were CD14+/CD16+. P24 antigen colocalized with both CD14 and CD16 suggesting that the CD14+/CD16+ macrophage is a major reservoir of HIV-1 infection in CNS. Using CD45/LCA staining, the perivascular macrophage was distinguished from resident microglia. In addition to perivascular and nodular localizations, CD16 also stained ramified cells throughout the white matter. These cells were more ramified and abundant than cells positive for CD14 in white matter. Double staining for p24 and CD16 suggests that these cells were often infected with HIV-1. The prominent distribution of CD14+ cells in HIVE prompted our analysis of soluble CD14 levels in cerebrospinal fluid. Higher levels of soluble CD14 (sCD14) were observed in patients with moderate-to-severe HIV dementia, suggesting the utility of sCD14 as a surrogate marker. CD14+/CD16+ monocytes may play a role in other neurological disorders and sCD14 may be useful for evaluating these conditions. *Journal of NeuroVirology* (2001) **7,** 528–541.

Keywords: HIV; CNS; dementia; microglial modules; monocytes

Introduction

HIV infection results in behavioral and motor disturbances, including dementia, in at least 20% of infected individuals (Navia *et al*, 1986; Gabuzda and Hirsch, 1987; McArthur *et al*, 1993) and almost all infected children with severe immunosuppression (Vazeux, 1991). In adult patients, the dementia is generally subcortical with loss of volume in the cortex and basal ganglia as determined by quantitative neuroimaging techniques (Aylward *et al*, 1993, 1995). Morphometric studies have demonstrated volume reductions in the cortical, subcortical, and white matter regions in HIV-infected adults and children (Kozlowski *et al*, 1997; Stout *et al*, 1998). Hallmarks of HIV-1 infection in the CNS include microglial nodules comprised of multinucleated giant cells as

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Abbreviations: CNS, central nervous system; HIVE, human immunodeficiency virus associated encephalopathy; MP, mononuclear phagocyte; SIV, simian immunodeficiency virus; LCA, leukocyte common antigen; CSF, cerebrospinal fluid.

well as other inflammatory cells. These lesions are found proximal to blood vessels and in areas distant from vessels, particularly in white matter, where they are associated with regions of white matter thinning and focal necrosis (Nielson *et al*, 1984; Sharer and Kapila, 1985; Budka *et al*, 1987; Sharer, 1992).

The mode of HIV entry into the CNS appears to involve trafficking of HIV-infected monocyte/macrophages from the circulation into the CNS (Meltzer *et al*, 1990). HIV-1 Tat protein, chemokine production, and adhesion molecule activation have been also implicated in the recruitment of additional monocyte/macrophages into the CNS from circulation (Schmidtmayerova *et al*, 1996; Bonwetsch *et al*, 1999; Nath *et al*, 1999; Liu *et al*, 2000; Wu *et al*, 2000). HIV-1 infection may initially spread to the CNS by a variety of mechanisms including trans-blood-brain-barrier migration of infected circulating monocytes, through infection or trans-cytosis of brain microvascular endothelial cells, through the blood-CSF barrier of the choroid plexus or alternatively by trafficking of T cells. The initial infection has been presumed to promote further cytokine and chemokine dysregulation, adhesion molecule activation, and impairment of the blood–brain barrier promoting an influx of additional activated monocytes, some of which are infected. An alternative hypothesis is that the process leading to HIV dementia begins outside the CNS, is dependent on the number of abnormal circulating monocyte subsets ready to invade the CNS, and occurs independently from the virus already in the CNS. In support of the latter model, the number of activated macrophages in the CNS is a better correlate of clinical HIV dementia than the level of virus in the brain (Glass *et al*, 1995). Previous studies demonstrating the accumulation of perivascular macrophages in the CNS in HIVE and SIVE (Rostad *et al*, 1987; Pumarola-Sune *et al*, 1987; Williams *et al*, 2001) as well as increases in abnormal monocyte subsets described in HIV infection provide further support. Increases in the population of CD14+ monocytes expressing CD16 have been observed in HIV infection (Dunne *et al*, 1996) and furthermore, increased numbers of circulating CD14+/CD16+ monocytes were observed in patients with HIV dementia (Pulliam *et al*, 1997). It has been proposed that alterations in the bone marrow cytokine environment may result in an increase in this activated monocyte subset that is more invasive with respect to the CNS compartment (Gartner, 2000). This model is consistent with phylogenetic analysis of viral quasispecies suggesting the trafficking of HIV-infected monocytes from the bone marrow to the brain (Liu *et al*, 2000).

Although the importance of the CD14+/CD16+ monocyte subset in HIV dementia was suggested based on increases in circulation in patients with HIV-associated neurological disorders, there have been no studies in human CNS tissue to directly examine this hypothesis. In the studies presented here, we provide clear evidence for CNS accumulation of CD14+/CD16+ cells, many of which are HIV infected, in HIVE.

Results

Utilizing a series of immunohistochemical markers that identify specific mononuclear phagocyte (MP) subsets, we investigated the role of the resident microglia and perivascular macrophages in the pathogenesis of HIV-1 infection in the CNS. A major issue in these studies and in the NeuroAIDS field is the ability to distinguish resident microglia from peripheral blood-derived macrophages in the CNS. Peripheral blood-derived perivascular macrophages, which are CD45+, can be distinguished from resting microglia, which are CD45−, by CD14 and CD45/LCA staining (Becher and Antel, 1996). CD14 is not normally found on resting microglia, but can be activated in cultured microglia. The level of CD45/LCA expression has also been used to distinguish human peripheral blood-derived macrophages from microglia. Expression of CD16, the Fc-γIII receptor, can be activated on leukocytes including peripheral blood-derived MPs and microglia (Ulvestad *et al*, 1994a, 1994b).

Using antibodies specific for CD14 and CD16, we examined the localization of CD14- and CD16-positive cells in brain tissue from 9 patients with HIVE, 4 patients with HIV-1 infection but without CNS disease, and 7 age-matched seronegative controls. The CNS- and non-CNS-related diagnoses of these patients are provided in Table 1. Relative to seronegative controls, there are increased numbers of perivascular CD14+ (Figure 1, compare Panels A and C) and CD16+ MPs (Figure 2, compare Panels A and C) in CNS of HIV-1-infected individuals with dementia (Table 2). Intermediate numbers of perivascular CD14+ and CD16+ MPs were observed in brain specimens from an individual with HIV-1-infection but without dementia (Figures 1 and 2, Panels B, and Table 2). Anti-CD14 (Figure 1, panel I) and anti-CD16 (Figure 2, Panel G) stained certain cells intensely within microglial nodules, particularly multinucleated giant cells. CD16 also stained ramified cells throughout the white matter in HIVE sections (Figure 2, Panel F and Table 2). In contrast, antibody to CD14 stained scattered ramified MPs throughout the white matter in HIVE sections, however the processes of these cells appeared to be shorter than those staining for CD16 (Figure 1, Panel G). CD14 staining was intense in multinucleated giant cells and in cells adhering to luminal surfaces of blood vessels (Figure 1, Panel I).

Cells within the microglial nodules also stained intensely for HLA-DR (Table 2 and Figure 3, Panel G). Some cells around blood vessels also stained positively (Figure 3, Panel C). Scattered stained cells with

Table 1 Patient data

Accession #	*Age/gender*	*HIV-1 status*	*CNS pathology*	*Non-CNS pathology*
MHBB 37	50 M	+	HIVE	Bacterial pneumonia, history of microspordia, CMV
MHBB 10070	37 M	+	HIVE	Bronchopneumonia, history of CMV retinitis
MHBB 10026	37 M	+	HIVE	*Klebsiella* pneumonia, sepsis
MHBB 76	38 F	+	HIVE	Bacterial pneumonia, CMV, ITP, MAI, PCP
MHBB 79	45 M	+	HIVE	Endocarditis, pneumonia, amyloid
MHBB 509	46 M	+	HIVE	Acute bronchopneumonia, cachexia, micronodular cirrhosis with jaundice
MHBB 500	47 M	+	HIVE	Wasting, candidal sepsis with foci in kidneys and lungs, urosepsis
MHBB 519	50 M	+	HIVE	Bronchopneumonia with diffuse alveolar damage
MHBB 10017	44 M	+	HIVE	Cachexia, esophagitis
MCPHU92-57	35 M	+	none	CMV pneumonia and cystitis
MCPHU96-105	41 M	+	Multiple infarctions w/diffuse intraventricular hemorrhage	Autopsy restricted to CNS
MCPHU98-20	32 M	+	CNS lymphoma	Autopsy restricted to CNS
MCPHU98-117	44 F	+	CNS lymphoma	Bronchopneumonia, liver cirrhosis
MCPHU99-14	34 M	−	Hypoxic/ischemic changes	Diabetes mellitus with end stage renal disease, bronchopneumonia
MCPHU99-19	69 M	−	Hypoxia	Acute MI, end-stage renal disease
MCPHU99-20	67 F	−	Hypertensive/diabetic vasculopathy	Coronary artery disease, hypertension, diabetes
MCPHU99-30	56 M	−	Hypertension, healed infarctions	S/P heart transplant, pneumonia, chronic renal failure
MCPHU99-38	22 F	−	Hypoxic/ischemic changes, acute infarction occipital lobe	Metastatic carcinoma, sepsis
MCPHU99-70	43 M	−	Hypertensive vascular disease, diffuse micro-infarctions	Endocarditis, pulmonary hypertension
MCPHU99-91	48 M	−	Hypoxic/ischemic changes	Hepatic failure

multinucleated morphology were also observed in white matter (Figure 3F).

Nonramified CD45/LCA+ cells were seen in HIVE sections predominantly within the perivascular region of vessels and within microglial nodules (Figure 4, Panels C and G), consistent with the peripheral blood-derived origin of the CD14+ and CD16+ MPs in this region. Occasional LCA+ cells were observed in the parenchyma, however these cells were small, nonramified, and likely to be monocytes or small lymphocytes. CD45/LCA staining of cells with MP morphology was rarely observed in parenchymal regions (Figure 4F) located beyond the perivascular cuff. This might suggest the limited potential of perivascular MPs to access the parenchyma. Alternatively, peripheral blood-derived monocyte markers are lost during differentiation and/or accommodation to surrounding CNS tissue. In support of this hypothesis, increased numbers of CD68+ cells were observed by immunohistochemistry in white matter in HIVE relative to control brain tissue (data not shown). The total number of macrophages/microglia therefore is increased, in agreement with an influx of perivascular MPs that adopt residency in the CNS.

Results with CD14, CD16, and CD45/LCA staining suggest the activated MPs associated with the microglial nodule and perivascular regions of vessels are perivascular monocyte/macrophage subsets and not resident microglia. The general absence of LCA staining of MPs in white matter suggests the possibility that CD16+ cells observed in white matter are resident microglia. The increased numbers of CD68+ cells in white matter in HIVE, however, suggest that the CD16+ cells in white matter accumulate from invading perivascular MPs that have taken on the phenotypic characteristics of microglia, with the loss of LCA and/or CD14 positivity.

Because increases in CD14+/CD16+ monocytes in circulation have been associated with HIV dementia (Pulliam *et al*, 1997), we performed CD14/CD16 double staining to determine if double-positive cells are present in CNS and to what extent these cells are associated with histopathologic abnormalities. By immunofluorescence double staining, CD14+/CD16+ cells were localized primarily within the perivascular space and within nodules in HIVE (Table 3 and Figure 5). To determine the localization of HIV-1 infected cells in CNS in HIVE, immunostaining was performed with anti-HIV-1 p24 antibody (7 cases, Table 2 and Figure 6). Anti-p24 antibody localized infected cells primarily around blood vessels, within microglial nodules and to a lesser degree in white matter. In four cases, the distribution of p24 staining

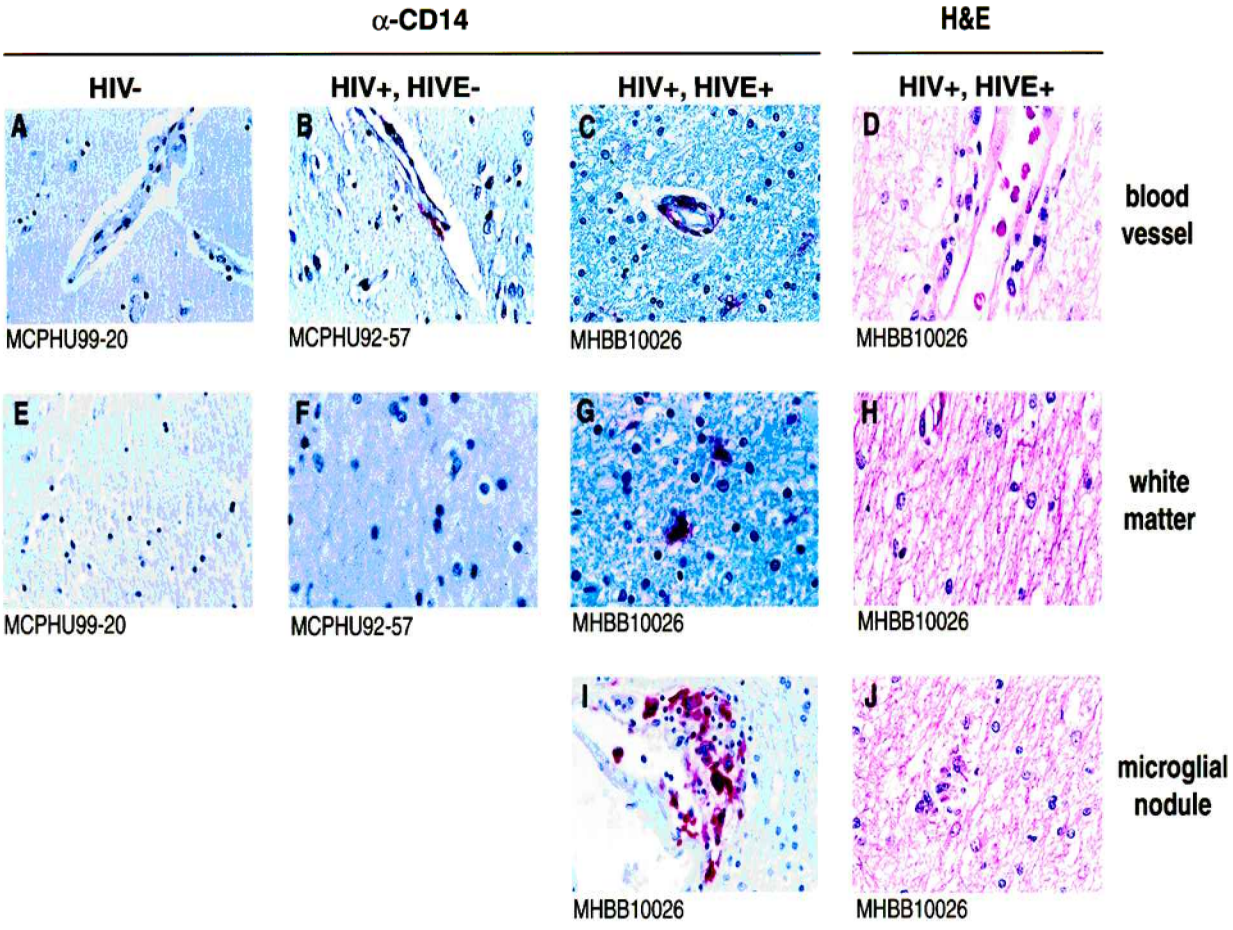

Figure 1 CD14 immunohistochemistry. (**A**) and (**E**), Normal Brain. (**B**) and (**F**) are HIV without encephalopathy. (**C**), (**G**), and (**I**) are from HIVE. Panels (**D**), (**H**), and (**J**) are hematoxylin and eosin-stained sections from the HIVE patient. The top row (**A**, **B**, **C**, and **D**) illustrates blood vessels. Middle row (**E**, **F**, **G**, and **H**) shows white matter. Panels (**I**) and (**J**) show microglial nodules.

was similar to the distribution of CD14 and HLA-DR. However, in two cases (MHBB 10070 and MHBB 509), there was limited p24 staining relative to the CD14 and HLA-DR positivity. In one case, there was actually more p24 positivity than observed with CD14 or HLA-DR staining (MHBB 10017). To further identify the subset associated with HIV-1 infection in CNS, we performed double staining of HIVE brain tissue specimens with anti-p24 and anti-CD14 or anti-CD16 in two of the cases (Table 3 and Figure 6). Intense p24 staining was observed in CD14+ MPs located primarily in the perivascular region and also in nodules (Table 3 and Figure 6). Perivascular CD16+ cells stained positively for p24 as did many ramified CD16+ cells in white matter (Table 3 and Figure 6).

In view of our results suggesting the increased accumulation of CD14+ perivascular MPs, as well as CD14+-activated microglia in white matter, we examined the level of soluble CD14 (sCD14) in CSF from a different set of patients with HIV-1 infection, HIV-1 infection with various degrees of dementia, and seronegative controls. ELISA assay of sCD14 in 16 HIV positive CSF samples and five seronegative controls demonstrated a statistically significant increase in sCD14 in CSF of HIV-1-infected patients with moderate-to-severe dementia (Figure 7). Although the mean sCD14 values were higher in HIV-positive than control and even higher in HIV-positive patients with mild dementia, these differences were not statistically significant. Future studies with larger groups may detect differences between control, HIV-1-infected and mild-to-severe dementia groups. It is interesting that one of the patients in the mild dementia group progressed to severe dementia over a period of few months; the sCD14 level doubled during this period, as determined by the analysis of the CSF sample obtained at the two visits (data not shown).

Discussion

HIV dementia and its associated histopathologic abnormalities are dependent on HIV-1 infection, but

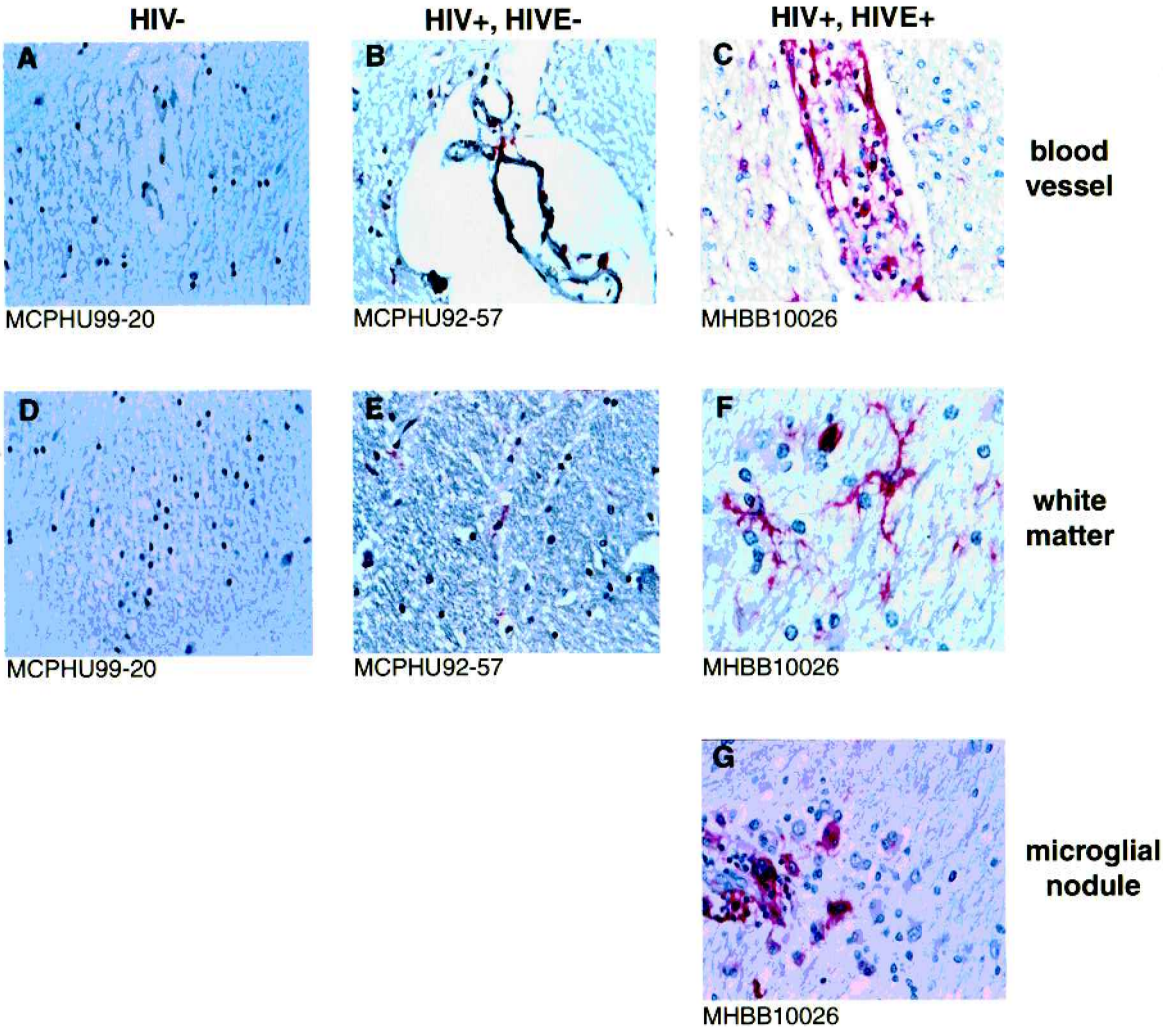

Figure 2 CD16 immunohistochemistry. (**A**) and (**D**), Normal Brain. (**B**) and (**E**) are HIV without encephalopathy. **C**, **F**, and **G** are from HIVE. The top row (**A**, **B**, and **C**) illustrates area of a blood vessel. Middle row (**D**, **E**, and **F**) shows white matter. Panel (**G**) shows a microglial nodule.

viral infection alone is not sufficient for the induction of CNS disease. The invasion of activated mononuclear phagocytes into the CNS appears to play a major role in the evolution of the disease process and the entry of HIV-1 into the CNS compartment. Although HIV-1 appears in the brain during acute infection, little virus is generated in or retained in the CNS until late in the course of disease (Davis *et al*, 1992; Bell *et al*, 1993; Donaldson *et al*, 1994; Teo *et al*, 1997).

In the process of HIV-1 invasion of the CNS, the perivascular macrophages appear to play an important role. HIV-1 has been localized to brain perivascular macrophages in HIV dementia (Pumarola-Sune *et al*, 1987; Rostad *et al*, 1987). Similarly, a recent study (Williams *et al*, 2001) identified the perivascular macrophage as the primary target of SIV infection in the CNS. Microglial nodules are usually localized in proximity to blood vessels, suggesting the role of the perivascular macrophage, rather than resident microglia in the formation of these lesions. Furthermore, increased numbers of perivascular macrophages are observed in the CNS in both HIV-1 and SIV infection (Power *et al*, 1993; Lane *et al*, 1996; Rostad *et al*, 1987; Price *et al*, 1988).

In the setting of HIV-1 infection, particularly in AIDS, alteration of monocyte activation status may be an important step leading to the development of neurological disorders. Increases in the percentage of CD14+/CD16+ monocytes have previously been demonstrated to occur late in the course of HIV-1 infection, and such alterations in circulating monocyte subsets are associated with dementia (Pulliam *et al*, 1997). This abnormal subset appears dense and granular and is capable of inducing neuronal apoptosis (Pulliam *et al*, 1997). The observation that the virus from brain in patients with HIV dementia most closely represents quasispecies in bone marrow

Table 2 Single label immunohistochemistry

Accession #	*CD14*	*CD16*	*LCA*	*HLA DR*	*p24*
HIV-1+, HIVE+					
MHBB 37	1+ PV	0	0	0	0
MHBB 10070	3+ PV 1+ WM 1+ nodule	4+ WM 2+ PV	1+ PV	3+ PV 3+ nodule	1+ PV 1+ nodule
MHBB 10026	4+ PV 1+ WM 2+ nodule	4+ WM 3+ PV	4+ PV 1+ WM	4+ PV 4+ nodule 1+ WM	ND
MHBB 76	1+ PV	2+ WM 1+ PV	2+ PV 1+ nodule	2+ PV 1+ nodule	1+ WM 1+ PV
MHBB 79	2+ PV 1+ WM 1+ nodule	1+ PV 3+ WM 1+ nodule	2+ PV 1+ WM 1+ nodule	3+ nodule 1+ PV	ND
MHBB 509	3+ PV 1+ WM 1+ nodule	2+ WM 1+ PV	2+ PV 2+ WM	1+ subventricular 1+ PV 1+ nodule	0
MHBB 500	3+ PV 1+ WM 2+ nodule	3+ PV 3+ WM 1+ nodule	ND	3+ PV 3+ nodule	2+ PV 1+ WM 2+ nodule
MHBB 519	2+ PV 2+ WM	4+ WM	1+ PV 1+ WM	1+ in region of WM thinning 1+ PV	2+ PV 1+ WM 1+ nodule
MHBB 10017	1+ PV	2+ PV 2+ nodule	3+ PV 1+ WM	3+ PV 3+ nodule	3+ PV 2+ WM 1+ nodule
HIV-1+, HIVE−					
MCPHU92-57	1+ PV	1+ PV 1+ WM	1+ PV	0	0
MCPHU96-105	0	0	1+ PV 1+ WM	0	0
MCPHU98-20	0	1+ WM	2+ WM	0	0
MCPHU98-117	0	1+ WM 2+ in areas of lymphomatous infiltration	2+ in areas of lymphomatous infiltration	2+ in areas of lymphomatous infiltration	0
HIV-1−					
MCPHU99-14	0	0	1+ acute inflammatory cells	0	0
MCPHU99-19	0	0	0	0	0
MCPHU99-20	0	0	0	0	0
MCPHU99-30	0	0	0	0	0
MCPHU99-38	0	0	0	0	0
MCPHU99-70	0	0	1+ acute inflammatory cells	0	0
MCPHU99-91	0	0	1+ acute inflammatory cells	0	0

PV = perivascular.
WM = white matter.

relative to other tissues further suggests the role of monocyte trafficking in the development of CNS disease (Gartner, 2000; Liu *et al*, 2000). In the CNS, both resident microglia and perivascular macrophages are derived from bone marrow. Microglial cells establish residence in the CNS during fetal development. Although little repopulation of resident microglial cells is thought to occur, perivascular macrophages are continuously repopulated from bone marrow (Hickey and Kimura, 1988; Lassman *et al*, 1993). It has been suggested that the increases in circulating CD14+/CD16+ monocytes may play a role in CNS invasion; however, there have been no studies in brain tissue to determine the presence of this abnormal monocyte subset in the pathogenesis of HIV dementia.

In the studies presented here, we examined brain tissue samples from HIV-1-infected individuals with dementia and without dementia and from seronegative controls. We found an abnormal accumulation of perivascular CD14+ and CD16+ cells in brain specimens from patients with HIV dementia. These cells appear to be derived from the circulation based on the large numbers of CD45/LCA+ cells localized perivascularly. Using double-fluorescence labeling immunohistochemistry, accumulation of CD14+/CD16+ cells was observed in the perivascular regions in brain tissue in HIV dementia. These results provide clear

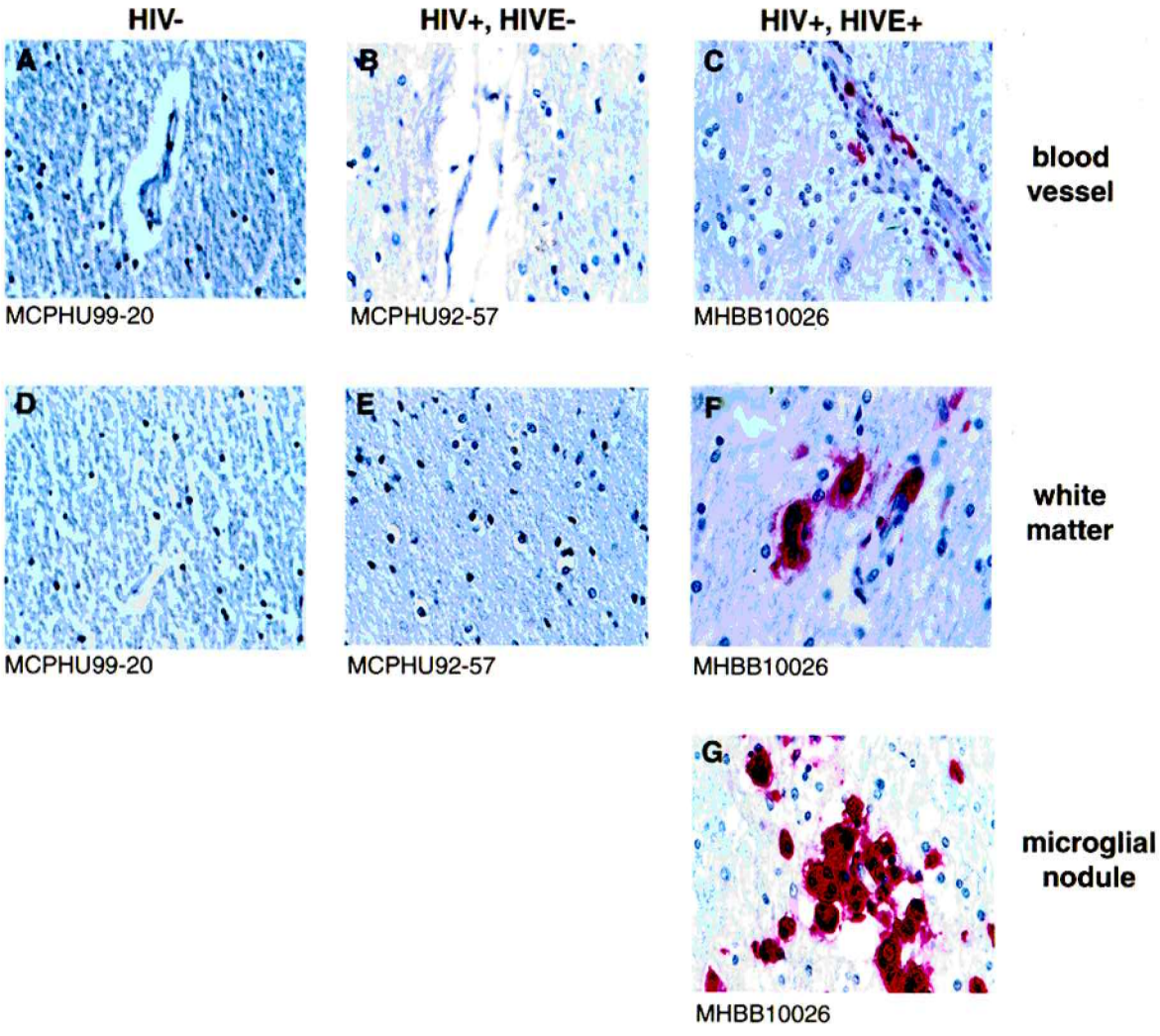

Figure 3 HLA-DR immunohistochemistry. (**A**) and (**D**), Normal Brain. (**B**) and (**E**) are HIV without encephalopathy. (**C**), (**F**), and (**G**) are from HIVE. The top row (**A**, **B**, and **C**) illustrates blood vessel. Middle row (**D**, **E**, and **F**) shows white matter. Panel (**G**) shows a microglial nodule.

evidence for the role of CD14+/CD16+ monocytes, previously found to be increased in circulation in HIV-1 infection, in the pathogenesis of HIV encephalopathy.

Our results with HIV-1 p24-immunostaining demonstrate that the CD14+ perivascular macrophage is the predominant cell type productively infected with HIV-1 in the CNS. HIV-1 p24-positive cells were localized proximal to blood vessels, in nodules, in the perivascular space, and to a lesser degree in white matter in 5 of 7 cases of HIV dementia examined. Using double-fluorescence labeling, HIV-1 p24 antigen was localized perivascularly, primarily within CD14+ cells in the perivascular region and in scattered ramified cells in the white matter. HIV-1 p24 also colocalized with CD16 staining in the perivascular regions, and to a lesser degree within white matter.

Viral load in the CNS is probably dependent on the perivascular accumulation of CD14+ cells, the percentage of CD14+ cells infected, and the general level of virus production. In 4 of 7 cases examined, the level of p24 staining in brain tissue correlated with the level of CD14+ cell accumulation in the perivascular region and in white matter. In two cases, there was an intense perivascular infiltrate, but with little p24 staining. These two cases may well have a lower percentage of infected perivascular cells. In one case, large amounts of p24 staining were evident, but with little CD14+ perivascular infiltrate. This case may represent the preferential infection of resident microglia with little perivascular cell invasion. Alternatively, the perivascular invasion may have resolved prior to death, leaving behind the infected resident microglia. The predominance of the perivascular cell accumulation in HIVE may explain the observation of severe clinical dementia in children with little evidence of HIV replication in CNS (Vazeux *et al*, 1992; Lacroix *et al*, 1993).

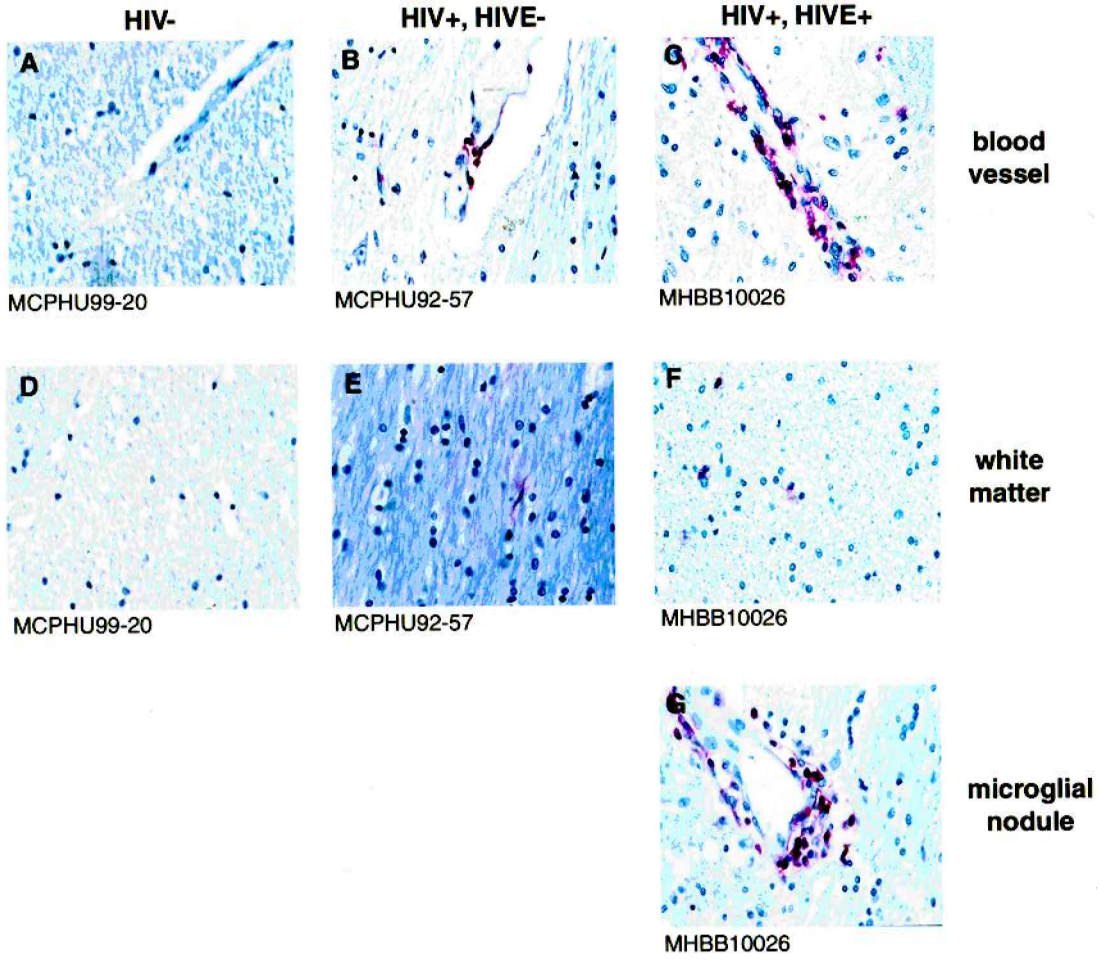

Figure 4 CD45/LCA immunohistochemistry. Panels (**A**) and (**D**), normal brain. Panels (**B**) and (**E**) are HIV-infected without encephalopathy. Panels (**C**), (**F**), and (**G**) are from HIVE. The top row, (**A**, **B**, and **C**) illustrates blood vessels. Middle row (**D**, **E**, and **F**) shows white matter. Panel (**G**) shows microglial nodule with juxtaposed blood vessel.

Although the CD14+ cells accumulating in the perivascular space appear to play a prominent role, the resident microglia may participate in the development of HIV-induced CNS disease. Our results might suggest that resident microglia are indeed activated as determined by CD16 positivity (Fc-γIII receptor expression). The substantive CD16 staining of ramified cells in areas where LCA staining is absent is consistent with activation of resident microglia (Figure 2, Panel F). Alternatively, these cells may represent invading CD14+/CD16+ monocytes that have lost CD14 expression upon differentiation and/or tissue accommodation. We have observed a substantial increase in CD68 staining in white matter in HIVE brain specimens (data not shown). It is therefore most likely that the origin of the CD16+ cells in the white matter represent a population of perivascular cells that have invaded CNS tissue and taken on the morphology of microglial cells.

Table 3 Double-label fluorescence immunohistochemistry

Accession #	*CD14/CD16*	*P24/CD14*	*P24/CD16*
MHBB 10026	3+ PV 1+ WM	ND	ND
MHBB 500	ND	2+ PV 1+ nodule	2+ WM 2+ PV
MHBB 79	2+ PV 1+ WM	2+ PV 1+ nodule	2+ WM 2+ PV

PV = perivascular.
WM = white matter.

Scattered CD14 upregulation within the white matter may also reflect resident microglial activation, or alternatively expression by peripheral blood-derived cells. There were few CD14+ cells relative to CD16+ in white matter in HIVE and the ramified processes of the CD14+ cells also appeared shorter. The ability of CD14 to be activated by *ex vivo* culture of resident microglia suggests that CD14 expression can be activated in the parenchyma, in addition to its association with invading monocytes (Becher and Antel, 1996). The role of activated resident microglia in neuronal dysregulation will require further investigation.

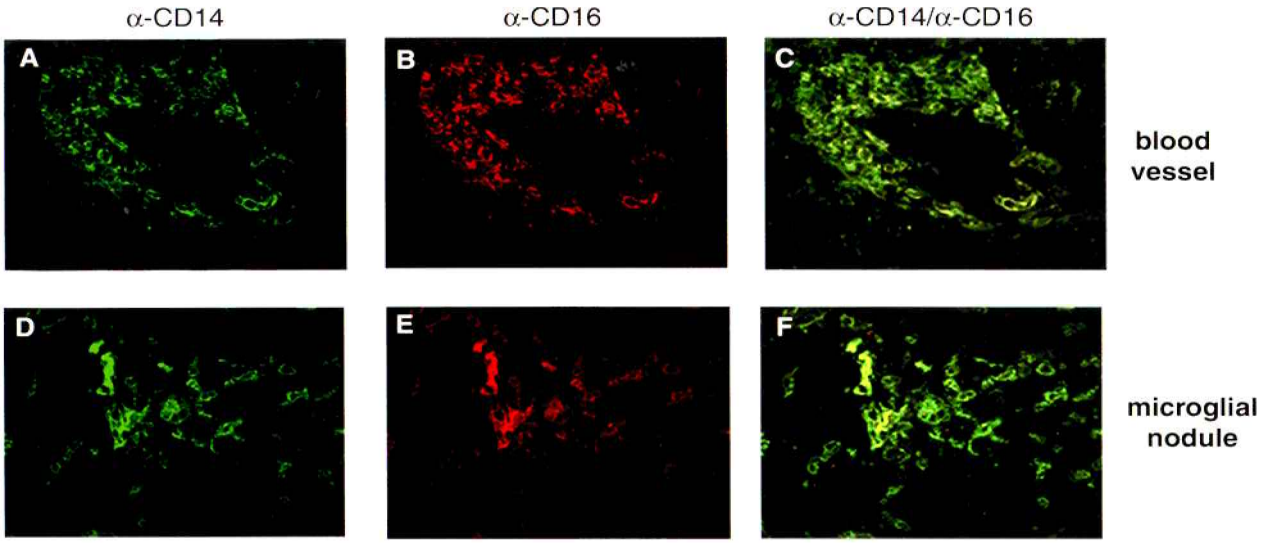

Figure 5 CD14/CD16 double immunofluorescence. In HIVE brain (Case # MHBB 79) CD14 intensely labels perivascular cells (panel **A**, fluorescein) as does CD16 (panel **B**, Texas Red). A large proportion is positive for both markers as demonstrated by yellow in the superimposed image (panel **C**). Microglial nodules also demonstrate CD14 (panel **D**, fluorescein) and CD 16 (panel **E**, Texas Red) expression with clear double positivity (panel **F**, yellow).

The notion that HIV dementia begins in the blood and/or bone marrow (Pulliam *et al*, 1997; Gartner, 2000) is supported by the observed efficacy of antiviral therapy in ameliorating CNS abnormalities. Successful HAART therapy results in improved motor and cognitive function and has reduced the incidence of HIV-associated neurodegenerative diseases (Brodt *et al*, 1997; Dore *et al*, 1999). In view of the inability of many of these drugs, particularly protease inhibitors, to cross the blood–brain barrier, the inhibition of viral replication outside the CNS presumably reduces the level of activated monocytes in circulation, thereby reducing monocyte trafficking and CNS invasion. A decrease in circulating virus, virus replication, or virus-induced cytokines may result in a decrease in monocyte activation.

CD14 is the receptor for bacterial endotoxin (LPS) (Wright *et al*, 1990). It is expressed on circulating monocytes, perivascular macrophages, Kupfer cells in liver, but not resident microglia in normal brain tissue (Ulvestad *et al*, 1994a). Increased numbers of CD14+ cells are observed in the CNS perivascular regions in certain inflammatory disease states including multiple sclerosis (MS). As a receptor for LPS, CD14+ cells can be activated by endotoxin to produce the inflammatory cytokines IL-6, TNF alpha, and IL-1 (Dentener *et al*, 1993; Gessani *et al*, 1993; Weidemann *et al*, 1994). This activity appears to involve intracellular signaling mechanisms through Toll-like receptor molecules (Yang *et al*, 1998). CD14 can be cleaved at the cell surface, resulting in a soluble form of the molecule (sCD14) capable of transducing effects of LPS in combination with LPS binding protein (LBP), to cells that normally do not express CD14. This activity may have important effects on endothelial cells (Pugin *et al*, 1993a, 1993b) including blood–brain barrier alterations and cytokine dysregulation. Increased levels of serum sCD14 levels are associated with HIV disease progression and the development of AIDS (Nockher *et al*, 1994; Lien *et al*, 1998).

In the studies presented here, we examined CSF levels of sCD14 in patients with mild dementia, moderate-to-severe dementia, and seronegative controls. Increased levels of sCD14 were observed in patients with moderate-to-severe dementia. These results are consistent with the accumulation of CD14+ cells perivascularly in HIV dementia and suggest a relationship between the level of CD14+ cell accumulation and the severity of dementia. Soluble CD14 may also play a role in the pathogenesis of HIV-induced CNS disorders. Increased levels of sCD14 in the CNS may promote increased sensitivity to bacterial LPS in patients with AIDS and may provide a mechanism for the onset or exacerbation of neurological abnormalities as a result of opportunistic infection. Alternatively, sCD14 may not contribute to the process of CNS disease evolution, but may serve as a useful surrogate marker for monitoring treatment of patients with HIV-induced CNS disease.

Further studies will be required to determine the mechanisms involved in the generation of the abnormal monocyte subsets and their mode of action in promoting CNS injury. It is tempting to speculate that perivascular accumulation of CD14+/CD16+ monocytes, with many harboring HIV-1, may not be limited to the CNS and may contribute to HIV-induced abnormalities in other organs and tissues, possibly contributing to immune dysfunction,

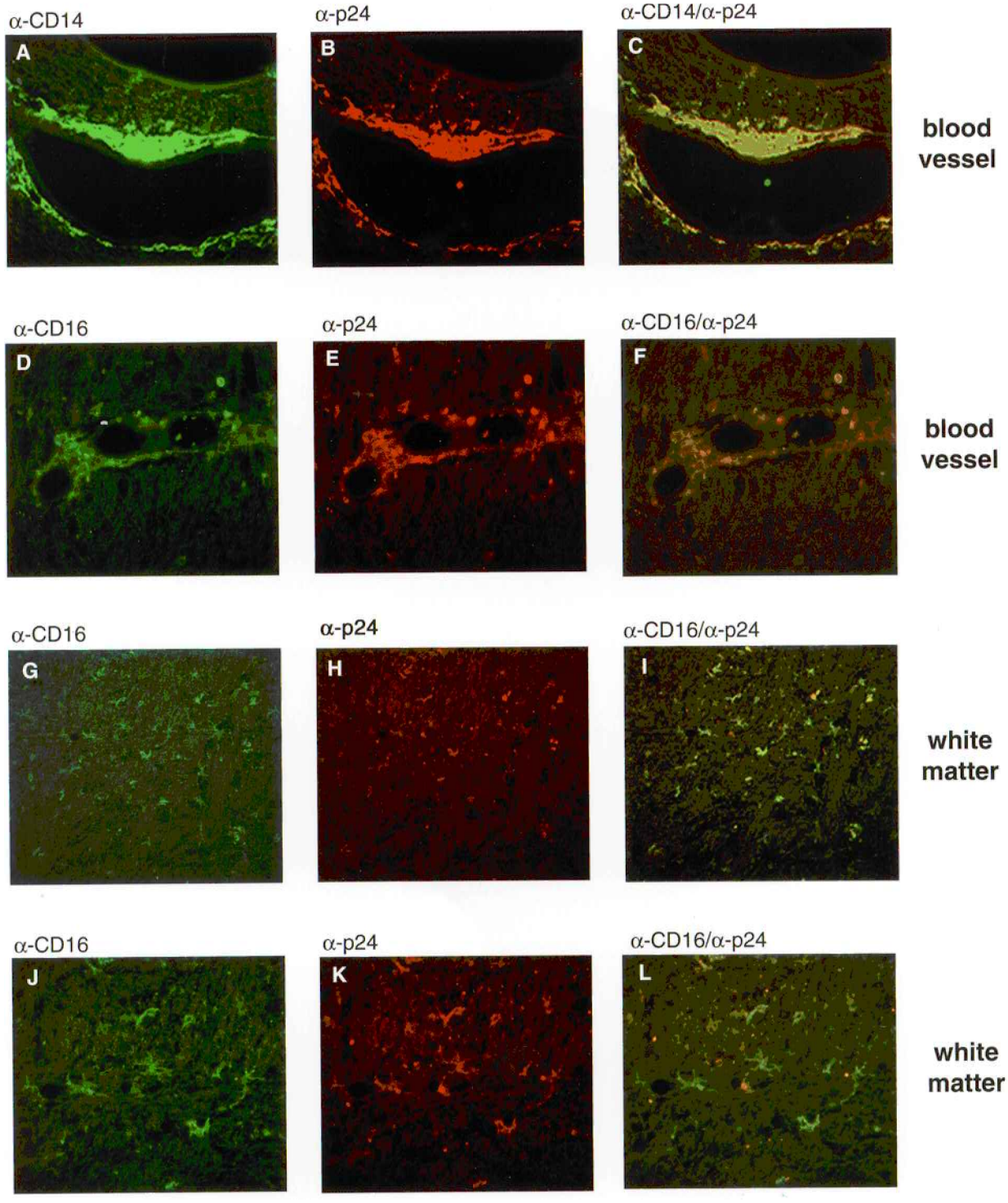

Figure 6 CD14/p24 CD16/p24 double immunofluorescence. In the same case as Figure 5 above (MHBB 79) CD14-positive perivascular cells (panel **A**, fluorescein) also show p24 positivity (panel **B**, Texas Red). Colocalization of these markers is demonstrated by yellow in the superimposed image (panel **C**). CD16-positive perivascular cells (panel **D**, fluorescein) also show p24 positivity (panel **E**, Texas Red). Colocalization is again demonstrated by yellow in the superimposed image (panel **F**). CD16-positive cells in white matter (panel **G**, low power, fluorescein; panel **J**, high power, fluorescein) are p24 positive as well (panel **H**, low power, Texas Red; panel **K**, high power, Texas Red) although the intensity of immunofluorescence is not as great as in the perivascular cells. Superimpositions (panels **I** and **L**) confirm the cellular colocalizations in yellow.

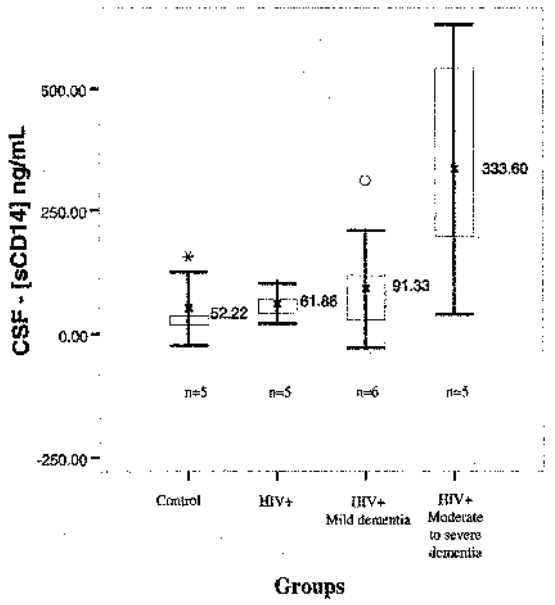

Figure 7 Analysis of soluble CD14 in patients with varying degrees of dementia. Results are shown for the following groups: Control (headache/seronegative), HIV without dementia, HIV with mild dementia, and HIV with moderate-to-severe dementia. Box plots show the mean for each group (x). Boxes contain the values from 25th to 75th percentiles; bars show the range of values. ∗ and ○ represent outliers. "n" denotes the number of samples in each group. Soluble CD14 levels were significantly higher in the moderate to severe group by ANOVA ($P = 0.016$).

renal, cardiac, intestinal, and pulmonary diseases in the setting of advanced HIV-1 infection.

Materials and methods

Human tissue samples

Paraffin embedded brain tissue samples (Table 1) from patients with and without HIVE were obtained from the Manhattan Brain Bank. Specimens from seronegative adults and HIV-positive adults without CNS disease were obtained from autopsy material from the MCP-Hahnemann University Autopsy Service. A total of nine HIVE cases, seven seronegative controls, and four HIV-positive cases with no apparent CNS disease were analyzed for CD14 and CD16 markers. A subset of these cases were evaluated for HIV p24 antigen, CD45/LCA, and HLADR.

Immunohistochemistry

Immunohistochemistry was performed by deparaffinizing 4-μ sections in xylene, followed by rehydration and nonenzymatic antigen retrieval in target retrieval solution (DAK0) at 95°C for 30 min. After cooling to room temperature (approximately 20 min), slides were rinsed in Tris-Buffered Saline with Tween (TTBS: 100 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4), followed by blocking in TTBS plus 20% normal horse serum for 2 h at room temperature. Slides were then incubated overnight with primary antibodies. To detect CD14, a mouse monoclonal antibody to human CD14 (Novocastra) was used at a 1:50 dilution. For CD16, a mouse monoclonal antibody to human CD16 (Novocastra) was used at a 1:40 dilution. A mouse monoclonal antibody to human HLA-DR alpha chain (Dako) was used at 1:100; mouse monoclonal anti CD45/LCA (Dako) was used at 1:25; and mouse monoclonal anti HIV-1 p24 (Dako) was used at 1:5 dilution. Positive controls were autopsy lymph node, liver, and lung samples from normal and HIV-1-infected individuals. Negative controls consisted of tissues incubated in buffer without primary antibody. Primary antibodies were detected with biotinylated anti-mouse secondary antibodies, avidin-biotin complex, and alkaline phosphatase–Vector Red according to manufacturer's instructions (Vectastain ABC AP Kit; Vector Laboratories). Endogenous alkaline phosphatase was quenched by the addition of levamasole (Vector) to the substrate solution. Following a light counterstain with hematoxylin, sections were dehydrated and coverslipped with Permount.

Scoring of sections: each section was scored blinded to diagnosis and patient identity but not blinded to antibody technique. Initial scoring was performed by two observers (SC and TF-S) independently. A score of 0 to ++++ was assigned to each section after the observation of a minimum of 10 microscopic fields at 40X objective magnification and was based on a qualitative average of the number of immunostained cellular profiles. Differences in the scoring by the two observers were resolved by reviewing the blinded slides together through a double-headed microscope.

Double-label immunohistochemistries for CD14/CD16, CD14/p24, and CD16/p24 were performed by sequential applications of primary antibodies to the same tissue section, which were both revealed by systems with different fluorescent tags. Following incubation of the first primary antibody and biotinylated secondary antibody, sections were incubated in fluorescein complexed to avidin (Vector fluorescein avidin ACS) at 1:100 dilution for 30 min. Tissue then was taken back to buffer and blocking reagent, incubated in the second primary antibody overnight, followed by secondary antibody and Texas Red complexed to avidin (Vector Texas Red avidin ACS) at 1:100 dilution for 30 min. Positive controls were autopsy lymph node, liver, and lung samples from normal and HIV-1-infected individuals. Negative controls consisted of tissues incubated in buffer without primary antibody, tissue incubated in one primary antibody, one secondary antibody, and one fluorochrome; and tissue incubated in one primary antibody but both secondary antibodies and both fluorochromes. The double-stained sections were examined on an inverted fluorescence microscope (Nikon) through both FITC and rhodamine filters. A digital photographic system (Princeton Instruments;

IP Lab) was used to superimpose images and evaluate the degree of colocalization of the fluorescent stained products.

CSF samples

CSF samples were provided by Dr. Avi Nath (Department of Neurology, University of Kentuckey, Lexington, KY) All CSF samples had been centrifuged after collection and cell-free CSF was aliquoted and immediately frozen at -70°C till further testing. Severity of dementia was categorized in the HIV-infected patients using the Memorial Sloan Kettering Scale (MSK). The patients were divided into three groups: No dementia (MSK = 0; $n = 5$), mild dementia (MSK = 0.5 or 1; $n = 6$), moderate to severe dementia (MSK = 2 or 3; $n = 5$). CSF from patients with headaches or degenerative disc disease ($n = 5$) were used as controls. Two samples in the HIV+ group (without dementia) were from the same patient taken at different times during the illness. Similarly, two samples in the HIV+ group with mild dementia were from the same patient at different times. These samples were treated as independent events for the comparison. One of the patients had samples entered into the mild dementia group and later into the moderate-to-severe group during the course of the illness. The former group is referred to as normal controls. CD4 counts and details of antiretroviral therapy were available on all patients. CSF viral loads were not available. CD4 counts (cells/mm^3; mean $\pm$ S.E.M.) were as follows. Nondemented group 342 ± 68; mildly demented group 268 ± 32; moderately to severely demented group 155 ± 71. Despite a trend for decrease in CD4 cell counts with the severity of dementia, the differences between the groups were not statistically significant. Only one patient in the moderately-to-severely demented group was on antiretroviral drugs, whereas four patients in each of the mildly and nondemented groups were on antiretroviral therapy.

Soluble CD14 ELISA assay

CSF samples were assayed in a blind fashion, without knowledge of HIV status or neurological condition. Assay was performed using Quantikine human sCD14 kit (R & D Systems). Samples were assayed in duplicate and results quantitated with standard curve ($R = 0.9748$). 100 μl of diluted CSF (1/20 dilution) was added to an equal volume of assay diluent in each well in a 96-well plate. Incubation was carried out for 3 h at room temperature. The plate was washed four times and 200 μl of sCD14 conjugate was added to each well. Incubation was carried out for 1 h and the plate was washed four times at room temperature. Substrate solution (200 μl) was added and incubation was carried out at room temperature for 30 min. After addition of the stop solution, O.D. readings were taken at 450 nm with wavelength correction at 570 nm. Differences between groups overall were evaluated using ANOVA. Mean differences were significant at $P \leq 0.05$ level.

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