RESEARCH ARTICLE

Human Brain Parenchymal Microglia Express CD14 and CD45 and are Productively Infected by HIV-1 in HIV-1 Encephalitis

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Microglia are endogenous brain macrophages that show distinct phenotypes such as expression of myeloid antigens, ramified morphology, and presence within the neural parenchyma. They play significant roles in a number of human CNS diseases including AIDS dementia. Together with monocyte-derived (perivascular) macrophages, microglia represent a major target of HIV-1 infection. However, a recent report challenged this notion based on findings in SIV encephalitis. This study concluded that perivascular macrophages can be distinguished from parenchymal microglial cells by their expression of CD14 and CD45, and that macrophages, but not microglia, are productively infected in SIV and HIV encephalitis. To address whether parenchymal microglia are productively infected in HIV encephalitis, we analyzed expression of CD14, CD45 and HIV-1 p24 in human brain. Microglia were identified based on their characteristic ramified morphology and location in the neural parenchyma. We found that parenchymal microglia are CD14+ (activated), CD45+ (resting and activated), and constitute approximately two thirds of the p24+ cells in HIV encephalitis cases. These results demonstrate that microglia are major targets of infection by HIV-1, and delineate possible differences between HIVE and SIVE. Because productively infected tissue macrophages serve as the major viral reservoir, these findings have important implications for AIDS.

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Introduction

Since the first AIDS epidemic erupted 2 decades ago, the CNS has been recognized as an important tissue site for viral replication. HIV infection of the CNS produces a characteristic clinical and pathological entity referred to as the AIDS-dementia complex (ADC), in which productive infection of monocyte/macrophage lineage cells appears to trigger a neurodegenerative cascade (10, 26, 36, 40, 41). Since the introduction of HAART therapy, the changes in clinical, virological, and immunological parameters in HIV+ individuals have highlighted the importance of infected tissue as potential viral sanctuaries (51). Particularly, the role of tissue macrophages in the progression of HIV infection has been re-emphasized (38, 53). The CNS has resident macrophages called microglia, which appear to play a central role in the pathogenesis of CNS infection.

Microglial cells constitute a distinct glial cell population in the CNS (9, 13, 29, 43, 61). Unlike neurons and *macro*glia, microglia are of mesodermal (bone marrow) origin and seed the brain early during development. In the mature CNS, microglia are ubiquitously present in highly ramified forms ("resting" microglia) (11, 30). They sense changes in the CNS microenvironment and participate in the subsequent events leading to characteristic disease manifestations. The type and extent of microglial involvement (as well as monocyte involvement) are, in part, dictated by the rate and amount of tissue destruction. In HIV-1 encephalitis, the pathological substrate of ADC, both monocytes and microglia are involved in the disease process. However, defining the relative contribution of macrophages and microglia in diseased brain is hampered by the fact that, in humans, no single surface marker exists that separates microglia from monocytes (9). In this regard, bone marrow chimera studies have contributed significantly to our understanding of macrophage physiology in the CNS. The prevailing concept is that perivascular macrophages (also referred to as perivascular microglia or perivascular cells) are different from parenchymal microglia. They are replenished from the bone marrow and present antigen in vivo, while parenchymal microglial cells represent a stable endogenous brain macrophage population separate from the perivascular cells (19, 44, 57).

Because of the differences in the biology of the 2 CNS macrophage populations, elucidating their relative contribution to the disease process may be of considerable importance. For instance, microglial cells are long-lived and can possibly survive as long as the life span of an individual, and they may permanently reside within the CNS. Conversely, perivascular macrophages can

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Case #	Age/Sex	*HAD	HIV-1 Status	†Other illness	‡HIVE grade (MN/ MGC)	§p24 grade (F or D)	CD14 Perivascular	CD14 Parenchymal
HIVE 1	47 M	+	+	cachexia, sepsis	++	+++ (D>F)	+++	+++
HIVE 2	37M	+	+	Neurosyphilis	++++	++++(D>F)	++++	++++
HIVE 3	50 M	‖ND	+	pneumonia, ‖CMV	++	+++(D>F)	+	+++
HIVE 4	32 M	ND	+	pneumonia, CMV	±	+ (D>F)	ND	ND
HIVE 5	33 F	ND	+	Kaposi's sarcoma, CMV, cachexia	+ MGCs)	+ (Isolated	+	+++
HIVE 6	45 M	ND	+	endocarditis, pneumonia, amyloid	+	++ (F>>D)	+	++ (MN only)
HIVE 7	31 M	+	+	Not available	+++	++ (F>>D)	+	+++
HIVE 8	44 M	+	+	cachexia, esophagitis	+++	++ (D>F)	++	+++
HIV+ 1	57 M	-	+	pyelonephritis, diabetes, heart failure, hypertension	-	-	+	+++
HIV+ 2	52 M	-	+	‖PCP, CMV	-	-	+	-
HIV+ 3	33 M	-	+	Colonic polyps, pneumonia, bowel resection	-	-	+	+
HIV+ 4	36 M	-	+	PCP, TB lymphadenitis, myocardial infarction	-	-	+	++
HIV+ 5	41 M	-	+	cachexia, PCP, ‖CHF	-	-	+	-
HIV+ 6	40 M	-	+	endocarditis, pneumonia, pyelonephritis, seizure	-	-	ND	ND
HIV+ 7	39 M	+	+	PCP, herpes, esophagitis	-	-	++	++
HIV- 1	56 F	-	-	scleroderma, candida esophagitis, cachexia	-	-	+	-
HIV- 2	57 M	-	-	s/p liver transplant for hepatocellular carcinoma	-	-	+	-
HIV- 3	45 F	-	-	breast cancer, bowel resection, cardiomyopathy	-	-	+	-
HIV- 4	38 M	-	-	Thymic carcinoid with metastasis	-	-	+	-

* Presence or absence of dementia. Clinical diagnosis based on retrospective chart review at autopsy, except cases HIVE 1, HIVE 2, and HIVE 8, which were analyzed prospectively. HAD: HIV-1 associated dementia.
† Findings at autopsy.
‡ Analysis based on H&E stain; quantitation of microglial nodules (MN) and multinucleated giant cells (MGC).
§ F (focal) and D (diffuse) refer to the pattern of p24 immunostaining.
‖ Abbreviations: Not documented (ND); Pneumocystis carinii pneumonia (PCP); cytomegalovirus (CMV); congestive heart failure (CHF).

Table 1. HIV-1 p24 and CD14 immunoreactivity in HIVE and control brains.

leave the CNS and home to systemic organs, and there is a high turnover rate of these cells in the CNS. Infection of these different pools of CNS macrophages may affect disease development and progression. The extent to which parenchymal microglia harbor virus is also important from the therapeutic point of view, since these cells may serve as a viral reservoir.

Although several major studies of HIVE have demonstrated the presence of HIV antigen in macrophages and microglia (5, 8, 12, 16, 47), the majority has failed to address the relative contribution of perivascular macrophages and parenchymal microglia. Recently, Williams et al tried to examine this in SIVE by using purported cell-type specific markers. They concluded that perivascular macrophages, but not parenchymal microglia, express CD14 and CD45 and are responsible for CNS infection in SIVE/HIVE (62). To address this question in HIVE, we performed quantitative analyses of HIV-infected cells in human brain. We used HIV-1 p24 immunocytochemistry on paraffin-embedded autopsy tissue and showed that this method is sensitive and specific. We employed a combination of high-resolution microscopy and Nomarski interference optics, as well as labeling for endothelial specific antigen, von Willebrand factor (VWF), to determine the location (perivascular vs parenchymal) and the shape of the p24+ cells. Our results show that approximately two thirds of p24+ cells in each case belonged to microglia rather than macrophages.

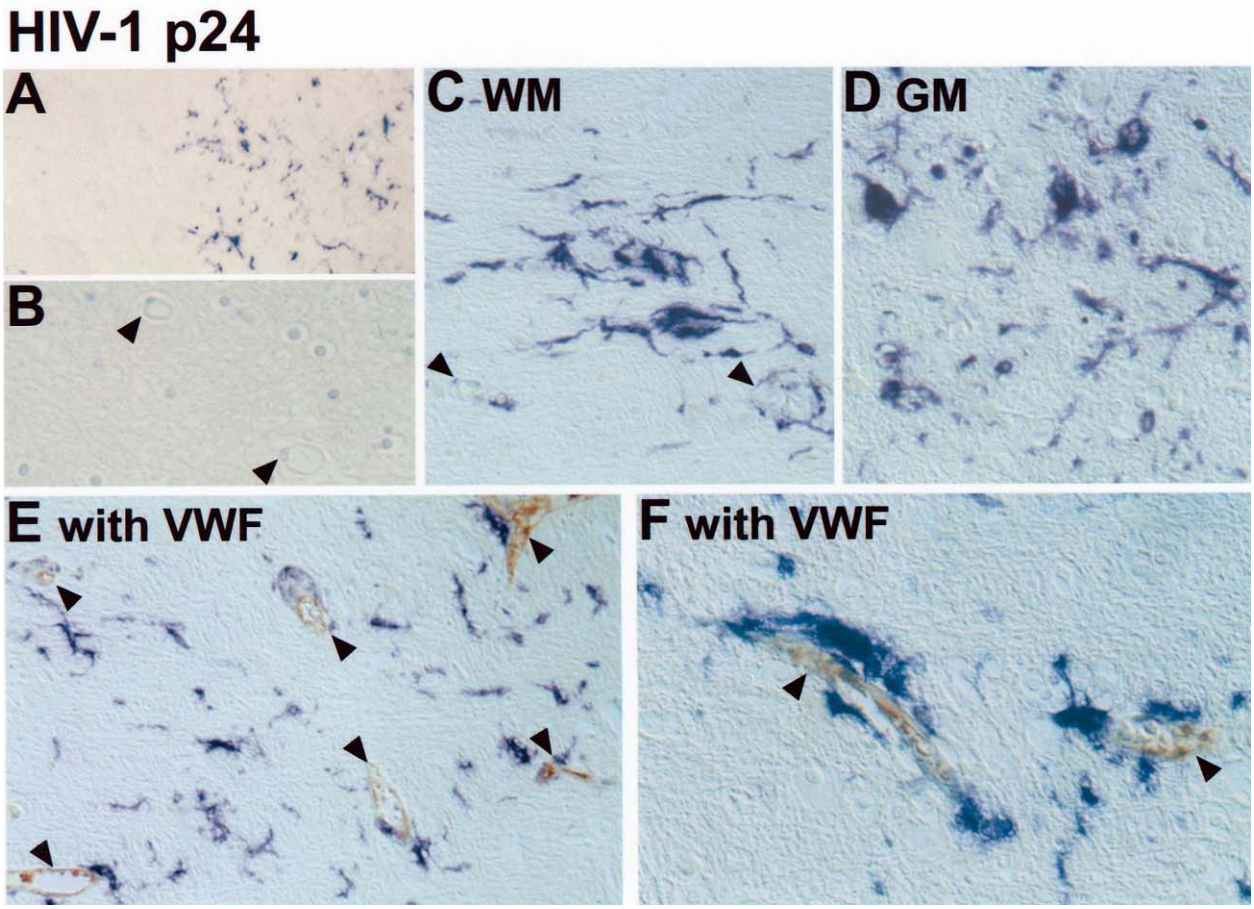

Figure 1. HIV-1 p24+ cells are found in both the parenchyma and the perivascular space in HIVE brains. (**A**) A low power view showing the diffuse distribution of p24+ cells (blue) interfacing an area with no p24 immunoreactivity. (**B**) p24 immunoreactivity was absent in non-HIVE cases, exemplified by this HIV-seropositive brain. Hematoxylin counterstained. (**C**) A white matter (WM) area displaying numerous p24+ bipolar cells. (**D**) A gray matter (GM) area displaying p24+ multipolar cells. (**E**, **F**) p24/Von Willebrand Factor (VWF; brown) double label immunocytochemistry. (**E**) A low power figure showing p24+ cells many of which have no relationship to the vessels. (**F**) Another area demonstrating the p24+ perivascular cells surrounding two blood vessels. Arrowheads point to vessels; vessels can also be visualized without VWF staining (**B**, **C**). Magnification: **A**, ×220; **B**, ×55; **C**, ×110; **D**, ×220; **E**, ×90; **F**, ×180.

Materials and Methods

Patient material. Paraffin-embedded, formalin-fixed brain tissues from 19 patients were obtained from the Manhattan HIV-1 Brain Bank. These consisted of HIVE (n = 8), HIV-seropositive without HIVE (n = 7) and HIV-seronegative individuals (n = 4)(Table 1). Brains with HIV-associated opportunistic infections or tumors were excluded in this study, except for patient 3 who had documented neurosyphillis. History was obtained by retrospective chart analysis at the time of autopsy, or collected prospectively. For each patient, one or two sections from the cerebral white matter and adjacent cortex or basal ganglia, each representing a region with observable HIVE pathology, was selected for immunostaining. Analysis of pathology was performed on hematoxylin and eosin (H&E)-stained slides.

HIV-1p24 immunocytochemistry. Deparaffinized sections were boiled in sodium citrate solution (DAKO: Carpinteria, CA) for 20 minutes for antigen retrieval. Sections were incubated with 3% H_2O_2 for 30 minutes, then with 10% normal goat serum for one hour. Mouse anti-HIV-1 p24 (IgG1; DAKO) was used at a concentration of 1:5 and incubated overnight at 4°C. The secondary antibody was either horseradish peroxidase (HRP)-labeled or alkaline phosphatase (AP)-labeled anti-IgG1. All secondary antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, Ala) and were used at a concentration of 1:200. To develop color,

Morphology	HIVE 1 Block A	HIVE 1 Block B	HIVE 2	HIVE 3 Block A	HIVE 3 Block B	HIVE 4	Mean ± SEM
1. Compact cells	5.2	5.8	6.3	6.7	9.6	4.8	6.4 ± 0.7
2. Macrophage-type cells	10.4	7.0	7.2	4.5	1.9	3.5	5.7 ± 1.2
3. †Process-bearing cells	8.6	6.3	6.5	6.6	6.4	6.0	6.7 ± 0.4
4. ‡MGCs without processes	0.6	0.5	1.4	0.0	0.5	0.7	0.6 ± .02
5. MGCs with processes	0.4	0.0	1.8	0.4	0.8	0.2	0.6 ± 0.3

*Average number of cells counted in 9-12 400× fields. See Figure 2 for illustration of each cell type.
†Process-bearing cells are mononuclear cells with one or more processes.
‡Multinucleated giant cells

Table 2. *Total number of p24+ cells classified by morphology.

either diaminobenzidine (DAB; brown) or 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; blue) alkaline phosphatase substrate (DAKO) was used for chromogen. When DAB was used, sections were counterstained with hematoxylin.

Double-labeling for von Willebrand factor (VWF) and HIV-1 p24. An anti-VWF antibody (rabbit; DAKO) was used at a concentration of 1:400 to label endothelial cells on deparaffinized slides that were subjected to antigen retrieval. An anti-rabbit HRP-labeled secondary antibody (DAKO) was used, followed by the chromogen (DAB, brown). Subsequently, staining for p24 was performed as discussed above, followed by incubation with an AP-labeled secondary antibody and color development with NBT (blue).

Immunostaining for macrophage markers: *CD14 and CD45.* Deparaffinized slides were boiled for antigen retrieval, then incubated with mouse anti-human CD14 (clone M-M42; IgG2a; Novocostra Laboratories, New Castle, UK) at 1:50 or CD45 (Leukocyte Common Antigen; clone PD7/26 & 2B11; IgG1; DAKO) at a concentration of 1:100 overnight at 4°C. Staining was completed using the tyramide signal amplification (TSA) Biotin System (NEN Life Science Products, Boston, Mass). We employed HRP-labeled secondary methods followed by exposure to tyramide solution according to the manufacturer's instructions. As a negative control, sections were incubated with mouse IgG2a or IgG1 isotype standard (BD Biosciences Pharmingen, San Diego, Calif).

Other immunohistochemistry. Staining for CD68 (KP-1: DAKO: 1:600) was performed as described 64. Additional markers for activated microglia and macrophages included IL-1a and interleukin-1 converting enzyme and the procedures for immunocytochemistry are as previously described (64).

HIV-1 p24+ cell counting based on morphology. P24+ cells were analyzed with respect to their shape and the numbers of cells belonging to each morphologic category were counted in twelve 400× microscopic fields (Figure 2; Table 2). They were classified into the following subtypes. *i)* "Compact cells" have a small round cell body and short knob-like processes. The majority of compact cells are located in the parenchyma. *ii)* "Macrophages" are rounded with abundant cytoplasm. They have an elongated cell body, especially when they wrap around the vessels. *iii)* "Process-bearing cells" have a single nucleus and one or more processes. Typical bipolar cells have an elongated cell body with two main processes, usually parallel to the direction of the fiber tracts, while the multipolar cells have multiple processes without directionality. *iv and v)* "Multinucleated giant cells (MGCs)" are those corresponding to the classic morphology of HIV-infected cells. They have a rounded cell body with or without processes. p24+ processes without visible cell body were not included in the cell counts.

HIV-1 p24+ cell counting based on location. To positively identify perivascular macrophages, we performed double-labeling with anti-VWF, an endothelial

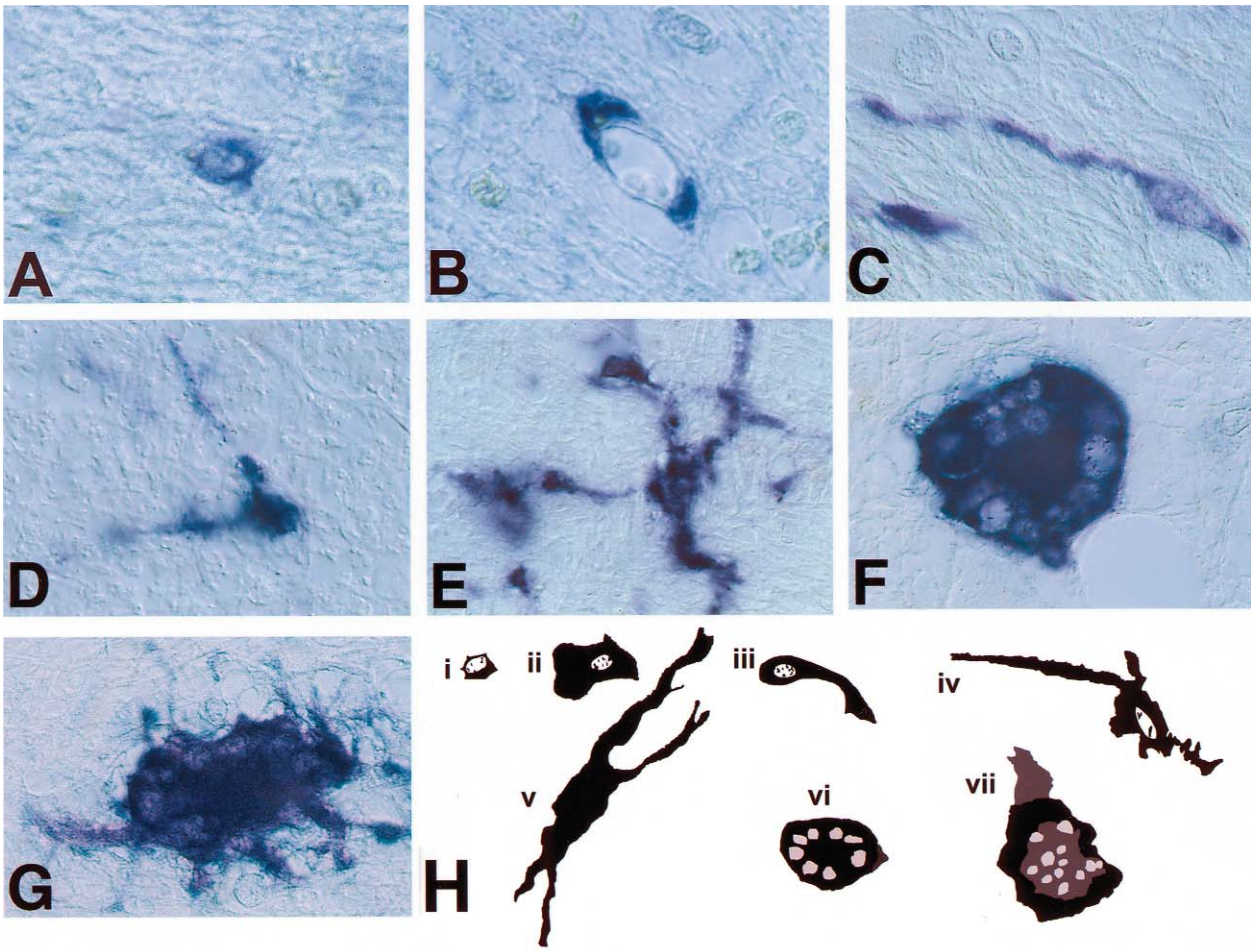

Figure 2. The variable morphology of p24+ cells. (**A**) a compact cell; (**B**) perivascular macrophages; (**C**) a unipolar mononuclear cell; (**D**) a bipolar cell; (**E**) a multipolar cell; (**F**) a rounded multinucleate giant cell (MGC); and (**G**) a process-bearing MGC. (**H**) Camera lucida drawings of p24+ cells: i. a compact cell; ii. a parenchymal macrophage; iii. a unipolar cell; iv. a bipolar cell; v. a multipolar cell; vi. a rounded MGC; and vii. a process-bearing MGC. Magnification: **A-G**, ×650.

cell-specific antigen (Figure 1E, F). Three cases of p24/VWF double-labeled slides were analyzed: cells directly adjacent to vessels or arranged in a linear fashion (implying a perivascular distribution) were classified as perivascular cells and cells that were haphazardly arranged within the brain parenchyma, without apparent relationships to vessels, were classified as parenchymal cells. Cells were counted in twelve 400× microscopic fields from 3 cases (Table 3).

HIV-1 p24+ cell density. To illustrate the distribution of p24+ cells, sections from 6 cases of HIVE were mapped according to the density of p24+ cells (4 shown, Figure 3). Sections were viewed at lower power (100×) and areas were marked according to cell density. Relative grades of p24+ cell density were used: 0, no p24+ cells (white); 1, low p24+ cell density (light gray); 2, intermediate p24+ cell density (dark gray); and 3, high p24+ cell density (black), as shown in Figure 3. Sections were then photographed and the areas with different p24 cell densities were outlined and color-coded with different shades using Adobe Photoshop®.

Camera lucida. Cells were reconstructed by pencil at 25× using a cameral lucida attachment on a light microscope (Carl Zeiss, Inc). Drawings were then scanned into the computer and Adobe Photoshop® was used to outline and fill in the cells.

Human fetal microglial culture and infection with HIV-1. Highly pure microglial cultures were generated as described previously (31, 33). Briefly, dissociated

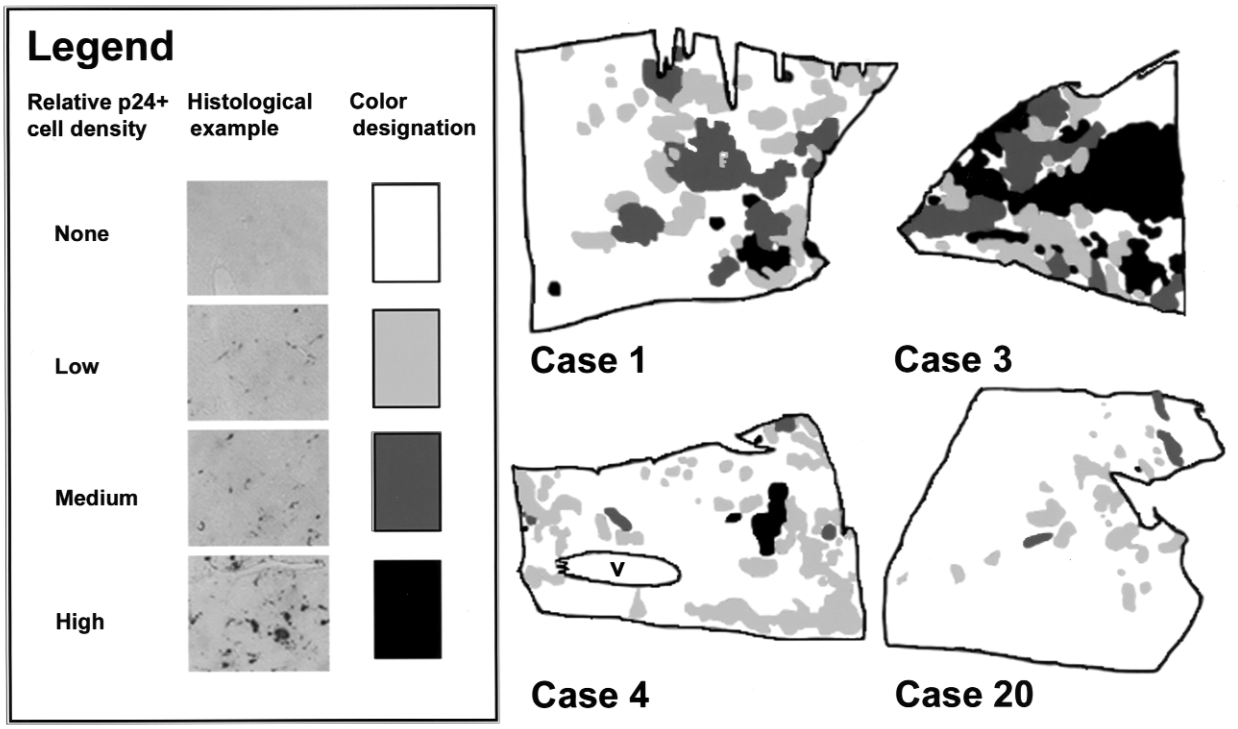

Figure 3. The density of p24+ cells in the cerebral white matter from four HIVE cases. Different shades, white, light gray, dark gray and black, represent different densities of p24+ cells in 4 brain sections: no p24+ cells, low density, intermediate density and high density, respectively. This is exemplified in the legend, which shows four images taken from a single case (HIVE 1). See the Materials and Methods section for a detailed description of methods. (v; ventricle.) Magnification for legend figures: ×55.

mixed CNS cell suspensions were plated as monolayers in DMEM/10% FCS. Approximately 14 to 21 days after plating, floating cells comprised primarily of microglial cells, were collected and re-seeded at a density of 40 000 cells per well in 96-well tissue culture plates. For viral infection, microglial cultures were exposed to HIV-1_{ADA} (obtained from ARRRP) at 2000 $TCID_{50}$ for 16 hours. They were then washed and fresh medium was added. Staining for p24, CD14, and CD45 was performed at 10 to 28 days post-inoculation, using methods as described above for brain tissues, except the antigen retrieval and dehydration steps were omitted.

*P24+ cell number	HIVE 1	HIVE 2	HIVE 4	Mean ± SEM
Perivascular cells	20.5	11.0	8.7	13.3 ± 3.6 (37%)
Parenchymal cells	27.5	20.9	20.1	22.8 ± 2.3 (63%)
Blood Vessels	9.8	10.1	8.7	9.5 ± 0.4

*Number of cells and bloods vessels are averages of those counted in twelve 400× fields.

Table 3. Localization of p24+ cells relative to vessels based on p24/VWF double-labeling.

Results

HIV-1 p24 immunocytochemistry demonstrates abundant staining in HIV-infected brain. HIV-1 p24 immunoreactivity was observed in 8 (89%) of 9 cases of HIVE and no p24 immunoreactivity was observed in control cases (Table 1; Figure 1; data not shown). The relative grade of p24 immunoreactivity ranged from low (+) to very high (++++) based on the *number* of positive cells within the *entire* section (Table 1). In general, the cases with very high p24 immunoreactivity were those with severe HIVE pathology based on H&E stain (MGCs and MNs). In addition, the distribution of p24 immunoreactivity ranged from focal to diffuse. Most cases showed a combination of the 2 patterns, but some showed p24+ cells confined to MNs (focal) (Table 1). p24 immunoreactivity can be observed in both the white and gray matter (Figure 1). Cells within diffusely stained areas had microglial morphology (Figure 1).

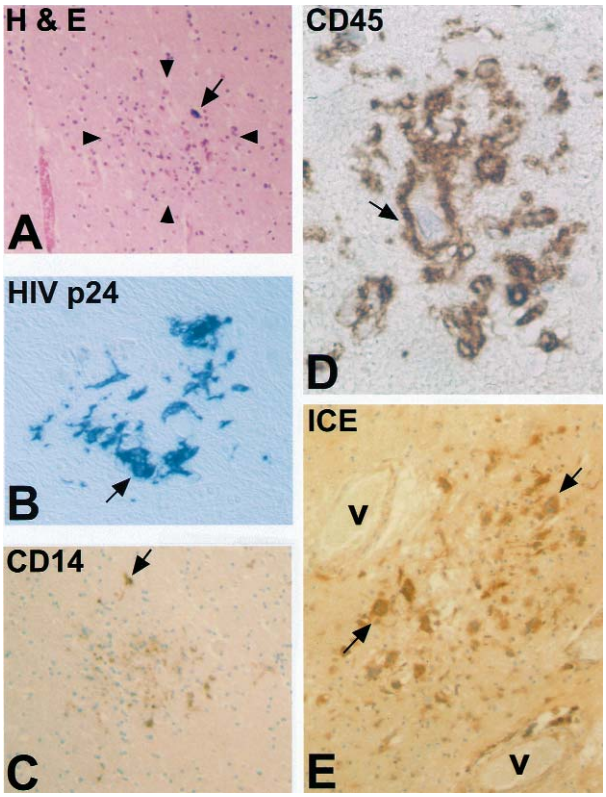

Figure 4. Microglial nodules (MN) in HIVE brain sections visualized with different histochemical and immunohistochemical methods. (**A**) H&E demonstrating a low power view of a MN in the cerebral white matter. MN is notable for focal hypercellularity, often with multinucleated giant cells (MGCs; arrow). Arrowheads mark the boundaries of the MN. (**B**) HIV-1 p24 immunoreactive cells comprise MN, here shown with Nomarski optics. NBT (blue) was used as a chromogen without counterstaining. P24+ cells are ramified in shape. Several MGCs are present, one is indicated by an arrow. (**C**) Low power view of an MN demonstrating CD14 staining of the constituent cells. This MN was found in the white matter. A MGC is evident (arrow). (**D**) CD45 intensely stains microglial cells in the MN. Note the membrane distribution of the immunoreactivity apparent on cell processes as well as a MGC (arrow). Note the lack of staining in the cytoplasm and multiple nuclei of the same MGC. (**E**) Cells of the MNs display many activation markers including IL-1 and its processing enzyme, ICE. Here shown with ICE immunostaining. Many MGCs (arrows) are present in this MN, which is localized between two vessels (v). DAB (brown) was used as the chromogen and all sections were counterstained with hematoxylin, producing purple nuclei (**C**, **D**, **E**). Magnification: **A-C**, ×210; **D**, ×410; **E**, ×210.

Analysis of p24+ cells. We used several different approaches in order to characterize p24-immunostained cells on paraffin sections. First, we performed detailed morphometric analysis of p24-labeled cells at 1000× magnification using oil immersion and Nomarski differential interference optics to facilitate visualization of unstained elements surrounding p24-positive cells. Second, we double-labeled sections for p24 and an endothelial cell-specific marker, VWF, to examine the location of p24+ cells with respect to vessels. Third, we analyzed the density and distribution of p24+ cells at low magnification to determine whether they correlated with the known distribution of macrophages or microglia

1. Morphology of p24-positive cells. The morphology of p24-positive cells varied a great deal and was consistent with that of macrophages and microglia. HIV-1 p24-positive cells were categorized and enumerated based on their shape, depending on 3 factors, ie, size of the cell body, presence or absence of processes, and the number of nuclei, as described in the Methods section. Examples of each cell type are illustrated in Figure 2. Of the subtypes of p24+ cells, microglial cells would have morphology consistent with that of compact cells, process-bearing cells (ramified cells), or process-bearing MGCs, while monocyte-derived macrophages would have the morphology of macrophages or non-process bearing MGCs. Compact cells were located within the brain parenchyma and have very small cell bodies compatible with microglial cells, but the attached processes were not visualized because the sections were only 4 to 6 micrometer-thick (Figure 2A). The morphologic subtypes of p24-positive cells were recapitulated by the Camera lucida drawings of p24+ cells (Figure 2H), and they demonstrate all the morphologic variants of microglial cells known to be associated with various locations and stages of activation (9, 30), as well as macrophages. Enumeration of p24+ cells in 6 sections from 4 HIVE cases demonstrated that approximately two-thirds of the p24+ cells in each case would belong to microglia based on morphology (Table 2).

2. Localization of p24-positive cells with respect to vessels. To reexamine the notion that perivascular macrophages and not parenchymal microglial cells are productively infected in SIVE/HIVE, we analyzed p24+ cells with respect to their localization, ie, perivascular versus parenchymal, based on the double-labeling of sections with antibody against an endothelial-specific marker, VWF, and p24 (Figure 1; Table 3). Vessels may also be visualized without staining (Figure 1B, C). Cells that were in direct contact with vessels or were arranged in a linear fashion resembling their distribution along the vessels, even when the vessels were not visible on the section, were counted as perivascular cells. Cells that did not exhibit either feature were counted as parenchymal cells. The results demonstrated that more of the p24+ cells in each case belong to the parenchymal

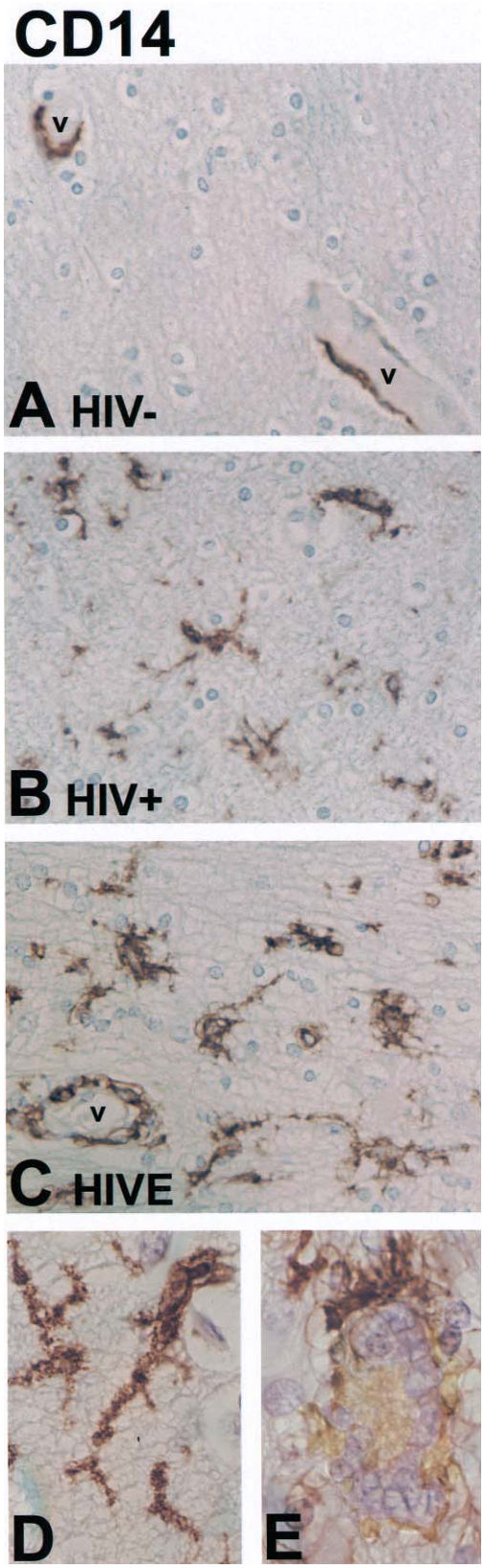

Figure 5. CD14 expression in parenchymal microglia is induced in HIVE. Sections from control or HIVE brains were immunostained for CD14. (**A**) HIV-seronegative brain showing CD14+ cells only in the perivascular space. Note the lack of staining in the parenchyma. (**B**) HIV+, non-HIVE brain showing some parenchymal microglial staining. (**C**) An HIVE brain showing robust CD14 staining on parenchymal microglia as well as perivascular macrophages. (v = vessel) (**D**) High power view of CD14+ microglial cells in HIVE. (E) High power view of a multinucleate giant cell showing weak CD14 staining, adjacent to a CD14+ process (top of the field), probably belong to another cell. Magnification: **A-C**, ×370; **E**, **F**, ×540.

microglia, with approximately two thirds of the p24+ cells belonging to parenchymal cells when all three cases were combined (Table 3). Therefore, the results were in good agreement with those generated based on morphology (Figure 2; Table 2).

3. Analysis of p24+ cell density. In order to document the distribution of p24 cells in a given section, we mapped the slides with respect to the density of p24+ cells using a 4-tier scale (ranging from none to high density) and generated a computer image of the section (Figure 3). There was a great variation in the density of p24+ cells from case to case and from area to area. Some areas showed no staining, others showed p24+ staining involving virtually all microglial cells within an area, with abrupt transition between these areas (Figure 1A). Because of the distance between vessels, the infection of perivascular cells alone would not generate the high-density of p24+ immunoreactivity. Rather, parenchymal microglial cells must be an unequivocal part of the infected population. This method of analysis reinforced the idea that parenchymal microglia are p24+ in HIVE.

Analysis of microglial nodules. Microglial nodules (MN) are a relatively specific pathology of HIVE, especially if they are combined with MGCs. The term clearly suggests the involvement of microglial cells, however, the notion that this may arise from the perivascular macrophages, rather than parenchymal microglia, was entertained. MN was a prominent feature in some HIVE cases, and the "focal" distribution of p24 generally corresponded to the MN type of pathology on H&E (Table 1). This was most notable in cases HIVE 6 and HIVE 7 where p24 staining was limited to the cells comprising MNs. The morphology of the constituent cells (ramified), the size of the nodules (50-700 mm), and the lack of endothelial marker (VWF), all point to the fact that microglial cells rather than perivascular macrophages comprise the MNs in HIVE (Figure 4). In addition to p24, MNs expressed monocyte/macrophage lineage markers, CD14 and CD45 (see below), and the monokine, IL-

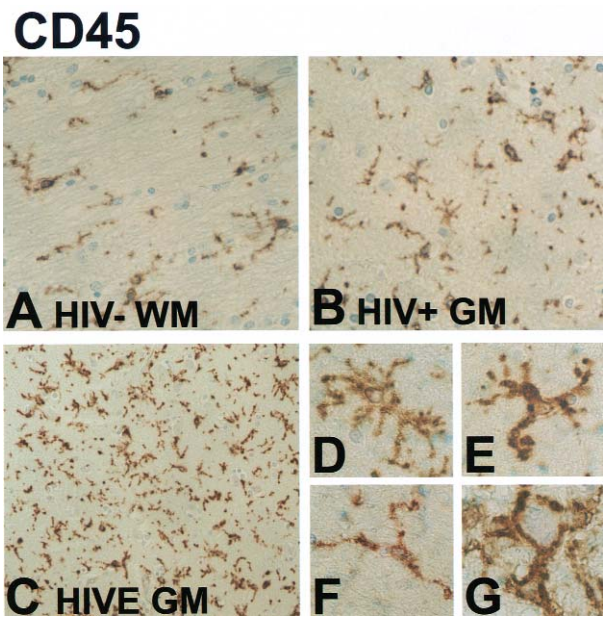

Figure 6. CD45 is constitutively expressed in microglia and is upregulated in HIVE. (**A**) White matter (WM) from a HIV-seronegative individual shows staining of delicate parenchymal microglia. (**B**) CD45 is also expressed in gray matter (GM) microglia in all cases, here shown in a case of HIV-seropositive control. (**C**) HIVE cases show an upregulation of CD45 expression in microglia. A section from cerebral cortex demonstrates strong immunoreactivity in many ramified microglial cells. (**D-F**) Higher power view of CD45+ microglial cells: extensively ramified cells in the gray matter (**D, E**), a ramified microglial cell in the white matter (**F**), and a multinucleate giant cell with intense CD45 staining on the membrane (**G**). Magnification: **A**, **B**, ×300; **C**, ×150; **D-G**, ×450.

1 (not shown), as well as its processing enzyme, interleukin-1 converting enzyme (ICE, casepase-1) (Figure 4) (64).

Expression of macrophage and microglial markers in HIVE and normal brain. We analyzed all of our cases for expression of CD14 because this was a putative marker for perivascular macrophages (56, 62) and because it was used to distinguish them from microglia in the Williams's study (62). The results are summarized in Table 1 and illustrated in Figure 5. Unlike previous reports, we found that CD14 was expressed on *both* microglial cells and macrophages, but the microglial expression varied depending on the state of activation. In HIV-negative control brains, CD14 expression was limited to macrophages in the perivascular and meningeal locations and parenchymal microglia were CD14-negative. In HIV-seropositive control brains, a spectrum of parenchymal microglial CD14 expression

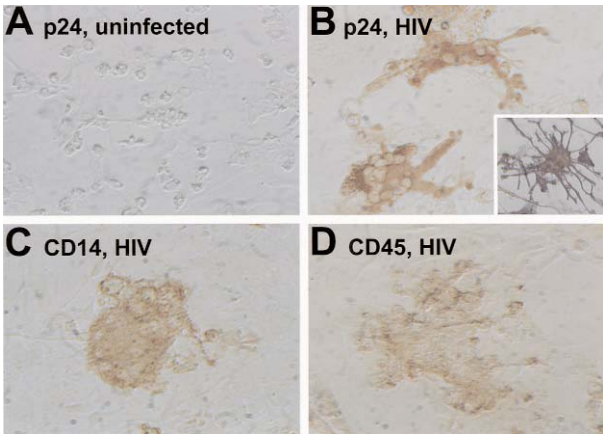

Figure 7. Cultured human microglial cells infected with HIV-1 recapitulate the pathology of HIVE. Human fetal microglia exposed to HIV-1_{ADA} are cultivated for 3 to 4 weeks and examined for the expression of p24, CD14 and CD45, by immunocytochemistry. Unlike uninfected control microglia (**A**), infected microglia show viral protein expression (p24) and syncytia (MGC) formation. The number of processes associated with MGCs in microglial cultures varies from none to few to numerous (**B-D**, inset B). MGCs are CD14+ and CD45+ (**C**, **D**), as are uninfected microglia (not shown). Astrocytes and occasional neurons in the infected culture are negative for p24, CD14, and CD45 (not shown). Cultures are immunostained as described in Methods, using DAB (brown) or NBT (blue; B, inset) as chromogen. Magnification: **A**, **B**, ×150 (inset ×130); **C**, **D**, ×150.

was seen ranging from normal levels to those seen in HIVE cases (Table 1; Figure 5). In HIVE cases, microglial CD14 expression was robust. However, not all microglia were CD14+; its expression coincided with areas of microglial infection and activation, as observed by p24 and CD68 immunostaining of adjacent sections (not shown). Interestingly, CD14 staining in MGCs was not uniform and ranged from none to weak to strong (Figure 5; data not shown). Perivascular macrophage staining was also robust in HIVE, as well as in some HIV+ control cases (Table 1; Figure 5; data not shown).

We also examined the expression of CD45, another presumed marker for perivascular macrophages (62), and found that it, too, was expressed in parenchymal microglia. Unlike CD14, however, CD45 was constitutively expressed in normal ("resting") microglia, in both gray and white matter, and its expression was further upregulated in microglia and macrophages in HIVE, including MGCs (Figure 6). In addition, infiltrating lymphocytes were strongly positive for CD45 (not shown).

Human fetal microglia are productively infected with HIV-1 in vitro. Human fetal microglia *matured* in

vitro in the presence of other neuronal cell types (especially astrocytes) display phenotypes characteristic of in vivo microglia. These include expression of monocyte/macrophage markers, morphology (ranging from ramified to ameboid), sensitivity to LPS stimulation, and ability to phagocytose (32-35, 37). They are also selective targets of productive HIV infection (25, 31). Similar to HIV-infected microglia in vivo, infected microglia in vitro exhibit process-bearing morphology (as well as "ameboid", macrophage-like morphology), p24-immunoreactivity, syncytia (MGC) formation, and are CD14+ and CD45+ (Figure 7).

Discussion

In the current study, we examined the cell types that are productively infected by HIV-1 in the human CNS, and showed that parenchymal microglia, in addition to perivascular macrophages, are productively infected by HIV-1. We performed quantitative analysis of HIV-infected (p24+) cells in HIVE cases and found that approximately two-thirds of the infected cells in the brain are microglia rather than perivascular macrophages. Since no macrophage markers are differentially expressed in the 2 populations, we employed morphologic criteria to identify microglia in our study. Our results corroborate earlier studies of HIVE by Kure et al and Budka et al, who found microglia to be a distinct subset of brain macrophages infected by HIV-1 (8, 27). Our results, however, are in stark contrast with a recent study of SIVE by Williams, which concluded that parenchymal microglia were not infected in SIVE/HIVE 62. All of the studies, including ours, found no evidence that non-macrophage lineage cells (ie, neurons, astrocytes, oligodendrocytes, and endothelial cells) were productively infected by HIV-1. The cell counts showed that MGCs represent only a minor fraction of p24+ cells in each case, demonstrating that H&E staining greatly underestimates the number of HIV-infected cells. Curiously, p24+ T cells were not detected, although T cells can be numerous in the brains of HIVE (60).

Our study delineated interesting histological features of HIVE with respect to the distribution of HIV antigen. Of the subtypes of HIV antigen distribution, the "diffuse" type is composed of parenchymal microglial cells. Our series exhibited this pattern in 5 (63%) of 8 cases. This is the type that is not found in SIVE and cannot be detected in routine histochemical analysis of the brain tissue. Analysis of the density of p24+ cells showed variable and irregular patterns inconsistent with the distribution of any known anatomic structures. Abrupt transitions were noted from very high to no p24+ cell areas. These results were inconsistent with the notion that HIV infection was restricted to perivascular macrophages.

Focal distribution or microglial nodules were the predominant pattern of infection in 2 (25%) of 8 cases. In contrast to the diffuse pattern, MNs were easily detected on H&E and consisted of activated glial cells and inflammatory cells. Activated ramified microglial cells were invariable components of MNs, as they displayed characteristic morphology, as well as expression of CD14, CD45, IL-1, ICE, and p24 (64). Although "glial" nodules are mentioned with regard to SIVE, they must be different in frequency and composition from MNs in HIVE, because the studies emphasize "perivascular" distribution of SIVE pathologies (62, 65). In addition, in our study, p24+ MGCs often displayed processes that cannot be appreciated with H&E staining. The location of MNs and MGCs in the brain parenchyma and the process-bearing morphology further support their microglial origin.

One of the phenotypic markers that Williams et al used to distinguish perivascular macrophages from parenchymal microglia was CD14 (62). CD14 is the LPS receptor and renders the cells exquisitely sensitive to the actions of LPS, as is the case for microglia (35, 55, 63). Additional functions of CD14 include regulation of apoptosis, apoptotic cell recognition, cytokine-mediated activation, and pattern recognition of non-LPS microbial components (18). A prior analysis of CD14 expression in human CNS by Ulvestad et al. (56) found that this antigen is exclusively expressed in macrophages and not in parenchymal microglia. In the current study, however, we report for the first time the expression of CD14 in activated parenchymal microglia. CD14 was detected in microglia of all HIVE cases and some HIV-seropositive control brains. In normal brain, CD14 expression was limited to perivascular and meningeal macrophages, with little or no expression in parenchymal microglia. The intensity of CD14 staining in microglia also correlated with the degree of microglial activation evident by their morphology. Therefore, our results support the notion that CD14 is expressed in all macrophages, but is induced in parenchymal microglial cells following activation and that our method of staining is sufficiently sensitive enough to recognize varying degrees of CD14 immunoreactivity. Interestingly, earlier studies by Becher et al reported the de novo expression of CD14 in cultured adult human microglial cells, alluding to the possibility that CD14 expression might be induced in microglia in vivo

(3). Together these results indicate that parenchymal microglia are immunologically downregulated macrophages that can be reactivated to express monocyte/macrophage lineage markers. They also demonstrate pitfalls associated with using antigenic profiles as sole criteria for distinguishing macrophages from microglia.

We also find CD45, another marker that the Williams' study found to be exclusively in perivascular macrophages, expressed in parenchymal microglia; this confirms prior studies in human CNS (24, 39, 57). CD45 is a transmembrane protein tyrosine phosphatase, and new evidence indicates its role in negative regulation of microglial activation and cytokine-receptor signaling (22, 42, 54). Expression of CD45 differs from CD14 because of its constitutive expression in ramified ("resting") microglial cells (24, 39). Its expression is upregulated in activated microglial cells in Alzheimer's disease and HIVE (39) (and this study). These data suggest that microglial CD45 may function to maintain the immunologically privileged state of the CNS and to downregulate inflammation following injury.

SIV models of AIDS provide invaluable tools for studying the pathogenesis and therapy of HIV-associated diseases, including CNS infection. Several different SIV isolates and clones can now generate CNS infection reproducibly in rhesus macaques and pigtail monkeys (45, 62, 66). The biology of SIV encephalitis (SIVE) has been studied in detail in several of these models. SIVE shows characteristics closely resembling those of HIVE, including the macrophage tropism of the virus and the pathology (MGCs, glial nodules, and widespread glial activation). Limited studies have investigated SIV antigen (gp41, p27 or gp120) distribution in these animals and reported a "perivascular" distribution of SIV-1 expression (45, 62, 65). A careful analysis of infected cell types is limited to 2 studies, and they remarkably describe the resistance of resident microglial cells to SIV infection in both early and late stages (4, 21). Although one cannot exclude that technical differences produce the different results in microglial infection in SIVE/HIVE, these data suggest that a distinct species-dependent difference in the microglial susceptibility to infection may exist.

Indeed, several important differences between SIV and HIV have been found, including the co-receptor usage. SIV and HIV share tropism for T cells and macrophages and utilize CD4 as well as CCR5 for viral entry. However, the vast majority of SIV isolates do not use CXCR4 for entry, one characteristic that distinguishes them from HIV-1 (20). In HIV-1 infected humans, a switch in the major co-receptor usage by HIV-1 from CCR5 to CXCR4 has been observed in about 50% of patients as they progress to AIDS, a change that can be associated with an increase in virulence and resistance to chemokine-mediated suppression (49, 58). In addition, in macaque models of SIV infection, progression from initial infection to AIDS takes only up to 1 to 2 years, in contrast to human HIV infection, where these events can take up to a decade to occur. It is conceivable that these differences could affect viral expression in the CNS, which typically occurs during the late phase of infection.

The paucity of documentation of microglial infection by SIV (7, 28) contrasts with the ease with which in vitro human microglia can be infected by HIV-1. The phenotype of human microglial cells in vitro closely resembles that in vivo. These cells exhibit remarkable plasticity including activation-dependent morphological changes ranging from highly ramified to ameboid (macrophage-like), as well as the potential to proliferate in response to GM-CSF (1, 15, 33-35). The latter distinguishes microglia from other tissue macrophages, and shows their plastic and uncommitted nature (48). We, as well as others, have shown that human fetal microglia, matured in vitro, can be productively infected by HIV-1 and display syncytia formation, virion production and p24/gp41 expression (17, 25, 31, 50) (and this report). Human microglia derived from adult brains also show characteristics similar to the fetal and in vivo human microglia (2, 23, 59). These studies therefore demonstrate that human in vitro systems can be used successfully to model the disease. Microglial culture systems provide invaluable tools for investigating virus-cell interactions, especially since monocytes are resistant to HIV infection, unless they are differentiated in vitro, often in the presence of growth factors (46).

The observation that microglia represent a major cell population productively infected by HIV-1 in HIVE has important implications. Because of their location in the brain parenchyma, where neurons and glia contact each other extensively through interdigitating processes, HIV-1 has a greater chance to interact with neurons and uninfected glial cells. This can be achieved through direct contact or indirectly via soluble viral/cellular products. Microglial cells represent tissue macrophages that permanently reside within the CNS, and are in a perfect position to serve as a viral reservoir. Recent findings suggest that HIV infection protects T cells and primary macrophages from apoptotic death (6, 14). We also observe that microglial cells expressing HIV-1 are pro-

tected from death (Cosenza et al, manuscript in preparation). These observations suggest that HIV-1 can find a true sanctuary within the CNS, and be a viral source for a low level, ongoing, productive infection in the periphery, as recently demonstrated in individuals on HAART therapy (52).

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