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Immunodetection of human immunodeficiency virus type 1 (HIV-1) Vpr in brain tissue of HIV-1 encephalitic patients

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Human immunodeficiency virus (HIV) encephalitis (HIVE), the most severe neurological complication associated with HIV-1 infection, leads to the onset of HIV-1–associated dementia (HAD). Several HIV-1 viral proteins have been implicated in HIVE-associated neurodegeneration. HIV-1 viral protein R (Vpr), a virion associated gene product known to induce apoptosis in nonproliferating cells, including neurons, is thought to contribute to the neuropathogenesis associated with HIVE. Though current research suggests that Vpr plays a significant role in neuropathogenesis, the presence of Vpr in the brain tissue of HIVE patients has not been assessed. Using a panel of HIVE patient brain tissue, the authors have shown that Vpr is present in detectable amounts in both the basal ganglia and frontal cortex of all HIVE brain tissue samples tested. Double immunofluorescence indicated that Vpr was found in the macrophages and neurons, but not in the astrocytes, of HIVE patients. These results for the first time show the presence of Vpr *in vivo* and further support the role of Vpr in neuropathogenesis. *Journal of NeuroVirology* (2006) **12,** 200–210.

Keywords: brain; encephalitis; HAD; HIV-1; neurons

Introduction

Human immunodeficiency virus (HIV)-1–associated dementia (HAD) is the most severe neurological complication associated with HIV-1 infection (McArthur, 2004). Pathological features leading to HAD include inflammation of the brain and neurodegeneration, which is defined as HIV encephalitis (HIVE) (Bell, 2004). Currently, 20% to 30% of people infected with HIV exhibit HIVE (Gonzalez-Scarano and Martin-Garcia, 2005). HIV-1 is believed to enter into the central nervous system (CNS) compartment via circulating infected monocytes that cross the blood-brain barrier and differentiate into macrophages. The CNS of HIV-1–infected individuals commonly contains HIV-1 antigen–positive macrophages and these infected perivascular cells have been shown to support a productive HIV (Koenig *et al*, 1986) and simian immunodeficiency virus (SIV) (Williams *et al*, 2001) infection within the CNS. HIV-1 virions released from the infected brain macrophages are believed to be taken up by other macrophages or astrocytes within the brain parenchyma (Liu *et al*, 2004). Additionally, infected macrophages release HIV-1 viral proteins and other neurotoxins that are hypothesized to be taken up by bystander cells within the brain parenchyma thus resulting in neuropathogenesis (Nath *et al*, 2000; Meucci *et al*, 1998; Hesselgesser *et al*, 1998).

Clinical manifestations of HIVE include widespread reactive astrogliosis, myelin pallor, and infiltration of monocytes that differentiate into brain macrophages (Budka, 1991). During HIVE, infected

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macrophages found within the CNS produce and release neurotoxins resulting in neuronal death (Lipton and Gendelman, 1995) and a subsequent onset of cognitive and motor deficits (Tenhula *et al*, 1992). The most severe neuronal loss and damage is seen in the frontal cortex of HIVE patients (Everall *et al*, 1991; Ketzler *et al*, 1990). HIV-1 encodes several proteins that disturb the cellular machinery of target cells within the CNS (Roshal *et al*, 2001), the most common form being apoptosis. HIV-1 viral proteins gp120, Tat, Nef, and Vpr are the viral proteins most often implicated in CNS cellular dysregulation (Yao *et al*, 2001; Shi *et al*, 1998; Rasola *et al*, 2001; Patel *et al*, 2000).

One of the HIV-1 viral accessory proteins, Vpr, has been implicated in contributing to neuropathogenesis. Vpr has been shown to be present in detectable amounts in the cerebrospinal fluid (CSF) of patients infected with HIV-1 (Levy *et al*, 1994). Extracellular Vpr forms ion channels across the planar lipid bilayers of the neuronal cellular membrane. These ion channels disturb the ionic gradient of the neuronal cells, causing a large inward cation current and depolarization of the plasma membrane, resulting in neuronal cell death (Piller *et al*, 1998). The amino-terminal region of Vpr that forms the ion channels that induces the neurotoxic effect (Piller *et al*, 1999). Vpr also induces apoptosis in human neurons through the activation of caspase 8 (Patel *et al*, 2000). This is significant because neuronal loss due to apoptosis is one of the hallmark features of HIVE (Everall *et al*, 1991; Ketzler *et al*, 1990). Additionally, Vpr up-regulates the proapoptotic molecule Bcl-2–associated death promoter (Bad) in neuronal cells (Patel *et al*, 2002), thus leading to neuronal apoptosis. Collectively, these studies indicate that Vpr plays a role in the cellular dysregulation of neurons, possibly inducing apoptosis via one or several mechanisms.

Despite the findings of previous studies suggesting that Vpr plays a significant role in the neuronal loss seen during HIV infection, no *in vivo* studies have been performed to identify the presence of Vpr in HIVE brain tissue. Through the use of immunohistochemistry, we have shown for the first time that Vpr is present in both macrophages (infected) and neurons in the brain tissue of eight HIVE patients. These findings further support the potential role of Vpr in HIVE-derived neuropathogenesis.

Results

Patients demographics, viral load, HIVE status, and tissue processing

A panel of brain tissue sections representing HIVE patients and HIV-seronegative controls were acquired for this study. The panel consisted of brain tissue from the frontal cortex and basal ganglia of eight HIVE patients, ranging in age from 33 to 47 years, who had died of acquired immunodeficiency syndrome (AIDS). Four seronegative age-matched controls were also analyzed. The patient demographics, viral load status, and other related information are summarized in Table 1. The median age range of the patient population is 40 years. This cohort included patients both undergoing highly active antiretroviral therapy (HAART) as well as patients no longer on therapy. However, in many patients HAART did not control viral load in either the serum or CSF, suggesting that these patients might be end stage.

Table 1 Patient demographics, viral load (plasma and CSF), and their HAART status

Patient ID	*HIVE status*	*Plasma viral load*	*CSF viral load*	*Age/ gender*	*HAART*
CA176	HIVE	ND	8,264	33M	Yes
CE129	HIVE	1,615,047	272,277	40M	Yes
CE144	HIVE	15,001	116,413	46M	No
ABBN 68-4041	HIVE	165682	365	35F	Yes
NNAB 2035	HIVE	1725	ND	44M	Yes
NNAB 2066	HIVE	250000	ND	34M	Yes
10017	HIVE	389120	>750000	44M	No
mhbb 500	HIVE	210000	ND	47M	No
CW01-072	Seronegative	NA	NA	57M	NA
CW01-073	Seronegative	NA	NA	48M	NA
CW00-148	Seronegative	NA	NA	62M	NA
CW99-100	Seronegative	NA	NA	54M	NA

Note. HIVE patients ($n = 8$) and HIV-seronegative patients ($n = 4$) were chosen for this study. Patients representing high and low viral load carriers, as well as HAART treated and HAART naïve, were included. ND, not determined; NA, not applicable.

Detection of HIV-1 p24 in HIVE brain tissue

HIV-1 p24 Gag is the most abundantly present antigen during HIV infection and previous studies have shown the presence of p24 in HIVE brain tissue (Cosenza *et al*, 2002; Fischer-Smith *et al*, 2001; Valle *et al*, 2002). HIV-1 Gag-p24 staining was performed in the tissue section from both infected ($n = 8$) and uninfected patients ($n = 4$) by immunohistochemistry to identify infected cells. Staining was performed in all HIVE ($n = 8$) and seronegative controls ($n = 4$) using specific p24 and control antibodies and a representation of two patients are presented in Figure 1. HIV-1 p24 was detected in the frontal cortex (Figure 1A and B) of the patients CE129 and NNAB2066, respectively. HIV-1 p24 staining was predominantly noted in macrophages (Figure 1A inlay) and microglia (Figure 1B inlay) of the frontal cortex. Further analysis indicated the cytoplasmic distribution of p24 in the brain macrophages coinciding with the pattern observed in HIV-1–infected target cells. Based on the intense staining found in the macrophages/microglia, it is possible to predict that these cells support active HIV-1 replication and viral protein synthesis in the CNS compartment. In the case of seronegative controls, there was no positive p24 staining in the frontal cortex (Figure 1C). In order to remove the possibility of nonspecific staining additional controls were performed using isotype control (Figure 1D). Results indicated that no nonspecific staining was occurring either with the Isotype control or with the secondary antibody, further

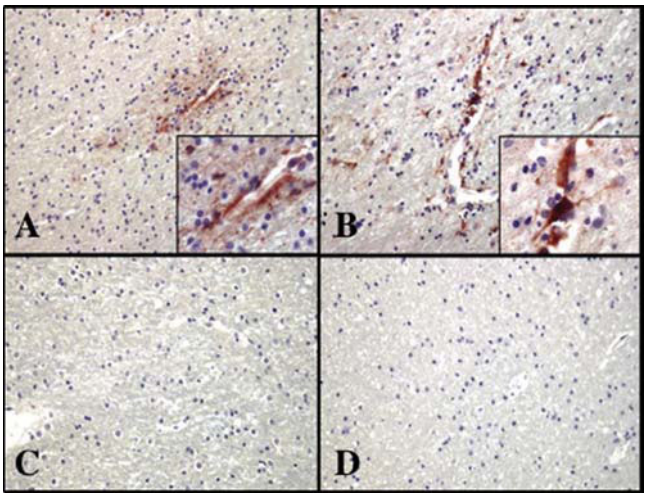

Figure 1 Detection of HIV-1 p24 in HIVE brain tissues. Detection of HIV-1 p24 in the frontal cortex of patients CE129 and NNAB2066 (**A** and **B**) of HIVE brain tissue. HIV-1 p24 staining was visualized with NovaRed peroxidase substrate (*red*), and the nuclei were counterstained with Mayer's Hematoxylin Solution (*blue*). Similar staining was frontal cortex (**C**) of seronegative control (CW01-073). Isotype control of the frontal cortex (**D**) for patient CE129 is represented here. Pictures were taken at 200× and inlays were at 600× magnifications.

confirming the validity of the p24 staining seen in Figure 1. Together these observations indicate the presence of HIV-1 p24 in CNS tissue of HIVE patients and further supports the finding that during HIVE, brain macrophages are the cells within the CNS that are most commonly infected by HIV-1, and can support productive HIV-1 infection (Koenig *et al*, 1986).

Optimization of HIV-1 Vpr staining in HIVE brain tissue

Despite the evidence from previous studies suggesting a role for Vpr in neuropathogenesis, attempts have not yet been made to detect Vpr in the brain tissue of HIV-positive individuals. Therefore it was necessary to optimize the conditions by which the tissue was treated in order to obtain accurate Vpr staining. Before using the Vpr antibody, the specificity of the Vpr antibody was tested using several methodologies, including immunoblot, immunoprecipitation, and immunofluorescence (Majumder *et al*, 2005; Thotala *et al*, 2004). These results indicate that the Vpr antibody used in our study specifically detected Vpr protein in all the tested assay systems, including transient transfection and infection systems. Furthermore Vpr antisera used for immunohistochemistry (IHC) detected recombinant (free), *de novo* synthesized (cell-associated), and virion-associated Vpr, indicating the specificity as well as the suitability of this antibody for this study. Because this Vpr-specific antibody was not used in immunohistochemistry assays before, conditions were optimized using five separate points in the technique: antigen retrieval, primary antibody incubation time and temperature, primary antibody dilution, secondary antibody incubation time, temperature, and dilution, and substrate exposure. Keeping four parameters constant while modifying the fifth, a protocol was developed resulting in clear and consistent Vpr staining that was used for Vpr staining.

Detection of HIV-1 Vpr in HIVE tissue by immunohistochemistry

Using the optimized protocol described above, all eight HIVE and four HIV-seronegative patient tissues were stained for Vpr. Results of the staining in three HIVE patients is presented in Figure 2A, as representative for all eight patients. The patients shown in Figure 2A are selected based on the availability of both the basal ganglia and frontal cortex tissue. As seen in Figure 2A, Vpr was detected in the basal ganglia (BG column; panels a, c, e) and frontal cortex (FC column; panels b, d, f) of the HIVE patients. Although only three representative patients (CA176, CE129, and CE144) are shown for the basal ganglia and frontal cortex, Vpr staining was observed in all eight HIVE patients in varying levels. The inlays within each panel exhibit the higher magnification (600×) of the positive cell. Based on cellular morphology, cells positive for Vpr in Figure 2A appear to be neurons (panels b and f), and brain macrophages (panels d and e).

Several negative controls were assessed to ensure the accuracy of the Vpr positive staining using the same patient tissue sections. The first control run was a no primary antibody control, which was run to show that the secondary antibody was not nonspecifically binding to the HIVE tissues. In performing the no primary antibody control set, the use of primary antibody step was omitted, and all other steps of the technique remained the same (Figure 2B; panels a, c, e). Results indicate that no specific staining was observed, which supports the accuracy of the Vpr staining seen in Figure 2A. Next, using the same tissue sections and a normal rabbit serum as primary antibody, we performed the staining. This control was included to rule out that the polyclonal Vpr primary antibody (raised in rabbit) did not react nonspecifically in the HIVE brain tissue. The tissue sections were incubated with normal rabbit serum in place of the primary antibody, at the same time, temperature, and dilution conditions (Figure 2B; panels b, d, f) and the results indicate no non-specific staining, further supporting the validity of the Vpr positive staining in Figure 2A.

Additionally staining was performed in all of the four HIV-1–seronegative, age-matched control tissues, using Vpr specific antibody (Figure 2C). Results presented here indicate that Vpr antibody did not bind to any of the seronegative samples further supporting the specificity of the Vpr antibody. Together these results indicate that the staining observed in HIVE patient tissues are Vpr-specific and support the presence of Vpr in the CNS compartment.

Comparison of Vpr and p24 staining in HIVE brain tissue

In order to assess whether there is a direct correlation between the number of cells positive for Vpr and p24, we quantitated the positive cells for each antigen. Tissue sections from each patient were scanned for 10 random fields at 400× magnification, and all the positive cells were counted. The sums for each field were then added together, and compared to a ranking scale (1–25 total positive cells = 1+; 26–50 total positive cells = 2+; 51–75 total positive cells = 3+; 76–100 positive cells = 4+). The summary of the results is presented in Table 2. When comparing the CSF and plasma viral loads (Table 1) to the number of cells positive for Vpr or p24, no apparent linear association was found. There was also no significant

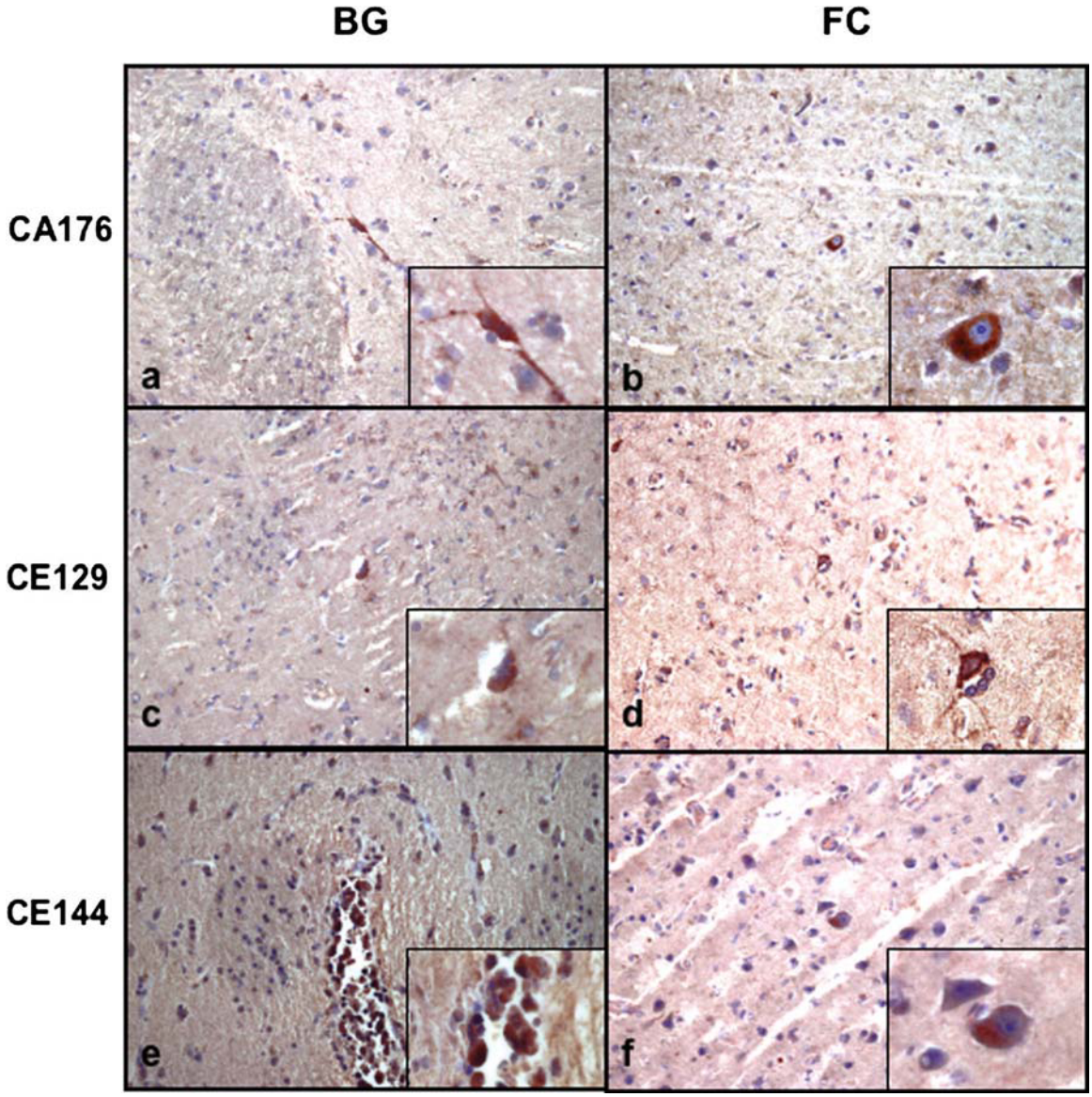

Figure 2A (**A**) Detection of HIV-1 Vpr by immunohistochemistry in tissue sections from HIVE patients. Immunostaining was performed using Vpr specific antibody as described in Materials and Methods. Three representative patients CA176 (a, b), CE129 (c, d), and CE144 (e, f) are shown here. BG, basal ganglia; FC, frontal cortex. HIV-1 Vpr staining was visualized with NovaRed peroxidase substrate (*red*), and the nuclei were counterstained with Mayer's Hematoxylin Solution (*blue*). Pictures were taken at 200× and inlays were at 600× magnification. (**B**) Immunohistochemistry controls for Vpr staining in seropositive samples. To test the specificity of Vpr antibody, additional no primary antibody (a, c, e) and normal rabbit antisera controls (b, d, f) were performed using the tissue sections of patients CA176, CE129, and CE144. Nuclei were counterstained with Mayer's Hematoxylin Solution (*blue*) and pictures were taken at 200× magnification. (**C**) HIV-1 Vpr immunohistochemistry in HIV seronegative specimens. HIV-seronegative controls were stained following the HIV-1 Vpr staining methods along with HIVE patients. BG, basal ganglia sections of seronegative donors CW01-072 and CW01-073. FC, frontal cortex section of patients CW00-148 and CW99-100. HIV-1 Vpr was stained and visualized with NovaRed peroxidase substrate and the nuclei were counterstained with Mayer's Hematoxylin Solution (*blue*). Pictures were taken at 200× magnification. *(Continued)*

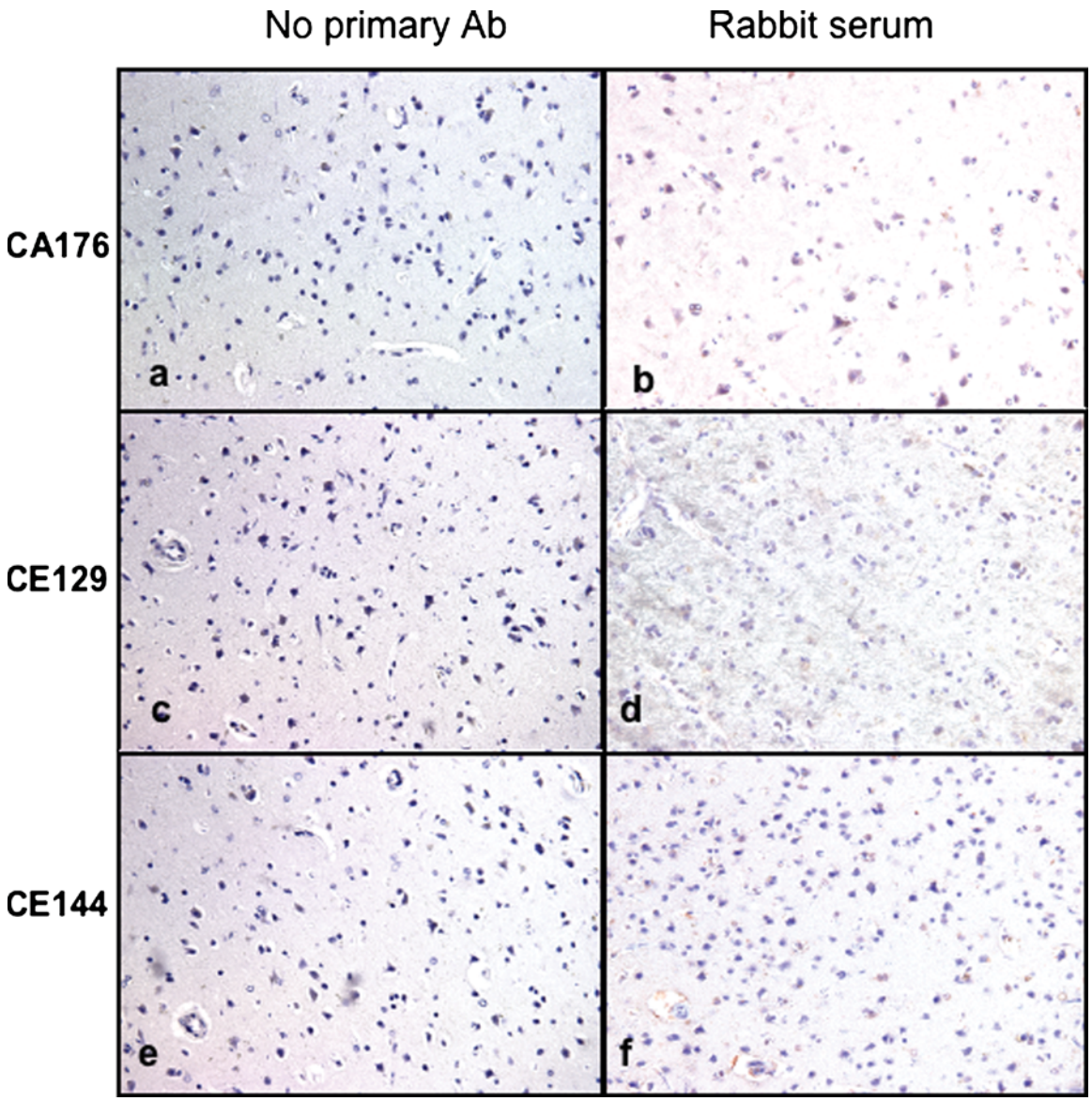

Figure 2B (Continued)

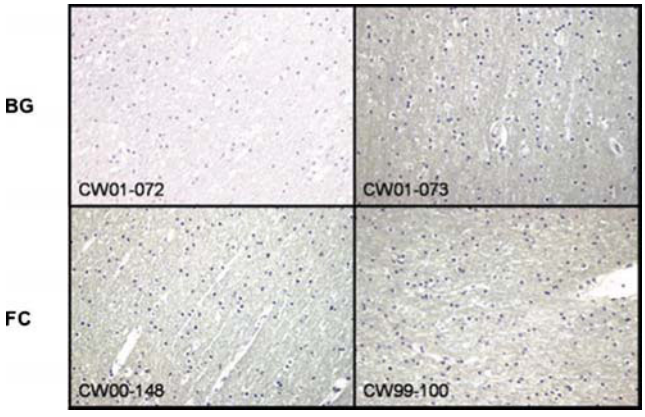

Figure 2C (Continued)

correlation observed between the number of cells positive for p24 and Vpr, regardless of patient history on HAART. This could be due to the single staining performed for each antigen separately instead of double immunohistochemistry, which was not possible due to the technical requirements involved in these staining protocols. However, the two patients with the highest CSF viral loads (CE129 and 10017) did exhibit the highest amounts of Vpr staining.

Detection of Vpr in specific cell types of present in HIV-positive brain tissue

Results presented in Figure 2A indicate the presence of Vpr in the brain tissue of HIV positive patients. In order to determine cell types positive for Vpr, we next performed double immunofluorescence

Table 2 Summary of Vpr- and p24-positive cells within the sections of brain tissue

Patient ID	*HIV status*	*Tissue Section*	*Vpr +*	*p24 +*
CA176-B	HIVE	Frontal cortex	3+	1+
CE129-B	HIVE	Frontal cortex	4+	4+
CE144-B	HIVE	Frontal cortex	2+	1+
ABBN 68-4041	HIVE	frontal cortex	2+	1+
NNAB 2035	HIVE	Frontal cortex	1+	2+
NNAB 2066	HIVE	Frontal cortex	2+	3+
CA176-G	HIVE	Basal ganglia	3+	1+
CE129-G	HIVE	Basal ganglia	3+	1+
CE144-G	HIVE	Basal ganglia	3+	1+
10017	HIVE	Basal ganglia	4+	2+
mhbb 500	HIVE	Basal ganglia	1+	3+
CW01-072-B	Seronegative	Frontal cortex	0	0
CW01-073-B	Seronegative	Frontal cortex	0	0
CW00-148-B	Seronegative	Frontal cortex	0	0
CW99-100-B	Seronegative	Frontal cortex	0	0
CW01-072-G	Seronegative	Basal ganglia	0	0
CW01-073-G	Seronegative	Basal ganglia	0	0
CW00-148-G	Seronegative	Basal ganglia	0	0
CW99-100-B	Seronegative	Basal ganglia	0	0

Note. Random fields ($n = 10$) for each tissue section were scanned at 400×, and all positive staining cells were counted. After adding together the sum from each of the 10 fields, the total number of positive cells was compared to a ranking scale. (1–25 total positive cells = 1+; 26–50 total positive cells = 2+; 51–75 total positive cells = 3+; 76–100 total positive cells = 4+.)

analysis using macrophage-, astrocyte-, and neuron-specific antibodies in the presence of Vpr-specific antibodies. Figure 3 (top row and middle row), exhibits Vpr-positive macrophages around the blood vessel in the basal ganglia in patients CA176 and CA144, respectively. Results also indicate that Vpr (green) is present in cells, which also stained positive for CD68 (red), a macrophage marker. Although both perivascular and brain macrophages can be seen adjacent to the brain microvasculature, they can be distinguished from one another based on morphology. Similar Vpr-positive brain macrophages were also observed in the frontal cortex of both the patients (data not shown). The seronegative control (patient CW01-072, basal ganglia) similarly stained (Figure 3, bottom row) demonstrating no Vpr in either the frontal cortex or basal ganglia, indicating that the staining described for Vpr and CD68 is not due to nonspecific staining of the conjugated secondary antibodies. Together, these results indicate that Vpr is present in detectable amounts in the infiltrating and macrophages of HIVE patients' brain tissue. Interestingly, we also observed an increased amount of brain macrophages in HIVE tissues compared to HIV-seronegative samples (data not shown) as previously shown by Glass *et al* (1995).

Detection of Vpr in astrocytes of HIVE brain tissue

Astrocytes are the most abundant cell type in the brain, and play a crucial role in brain homeostasis. Astrocytes have been shown to be susceptible to HIV infection through a CD4-independent, human mannose receptor (hMR)-dependent mechanism (Sabri *et al*, 1999; Liu *et al*, 2004). However, HIV-1 infection in astrocytes can only be detected through sensitive techniques that involve HIV-1 RNA or proviral DNA detection, suggesting that astrocytes are persistently infected rather than productively infected (Gorry *et al*, 2003; Tornatore *et al*, 1994). Based on the suggested role of Vpr in astrocytic cell death, it was important to determine whether Vpr was present in detectable amounts in astrocytes of the HIVE brain tissue. Double immunofluorescence was performed using an astrocytic marker, glial fibrillary acidic protein (GFAP), and the results are presented in Figure 4A for Patient CA176 as representative for the eight HIVE patient samples. Interestingly, no Vpr staining is observed within the cells that are positive for GFAP either in the basal ganglia (BG) or frontal cortex (FC). However, both the sections exhibit brilliant staining for GFAP as well as demonstrate astrocyte morphology, indicating the presence of astrocytes in these sections. Though Vpr staining is prominent around the blood vessel within these sections, Vpr fails to colocalize with GFAP, suggesting that Vpr is not present in the astrocytes of either the basal ganglia or the frontal cortex of HIVE patients. HIV-seronegative patients were used as negative controls, and run along side the HIVE patients in the double immunofluorescence for Vpr and GFAP. No Vpr staining was demonstrated in any of the brain tissue sections of the four seronegative controls (data not shown). It is not clear whether the levels of Vpr protein present in these cells are undectable or if astrocytes do not support robust virus replication, possibly due to their defeat in expressing late transcripts such as Vpr (Brack-Werner, 1999). Interestingly, we observed an increased number of astrocytes within the HIV-positive tissue sections (Figure 4B), indicating that HIVE-specific pathogenic features are observed in these tissues. This supports previous studies that demonstrate the metabolic activity of astrocytes increases in HIVE patients via a process known as astrogliosis (Sabri *et al*, 2003).

Vpr detection in neurons of HIVE brain tissue

Several groups have shown that HIV-1 viral proteins can cause injury or death of neurons in the absence of direct infection (Nath *et al*, 2000; Meucci *et al*, 1998; Hesselgesser *et al*, 1998), implying that HIV-1 proteins could be taken up by neurons. Extracellular Vpr has been shown to transduce through intact cytoplasmic membranes into cells that are not normally infected by HIV-1 and cause cell death (Sherman *et al*, 2002). To determine whether Vpr is present in detectable amounts in the neurons of HIVE brain tissue, double immunofluorescence was performed using antibodies specific for the neuronal marker, SMI312, and Vpr. Figure 5 demonstrates the presence of Vpr-positive neurons in both the basal ganglia (BG) and frontal cortex (FC) of patient CA129. Neuronal marker SMI312 exhibited cytoplasmic localization in neurons, whereas Vpr-positive staining was seen on both the cell surface and in the nucleus of SMI312

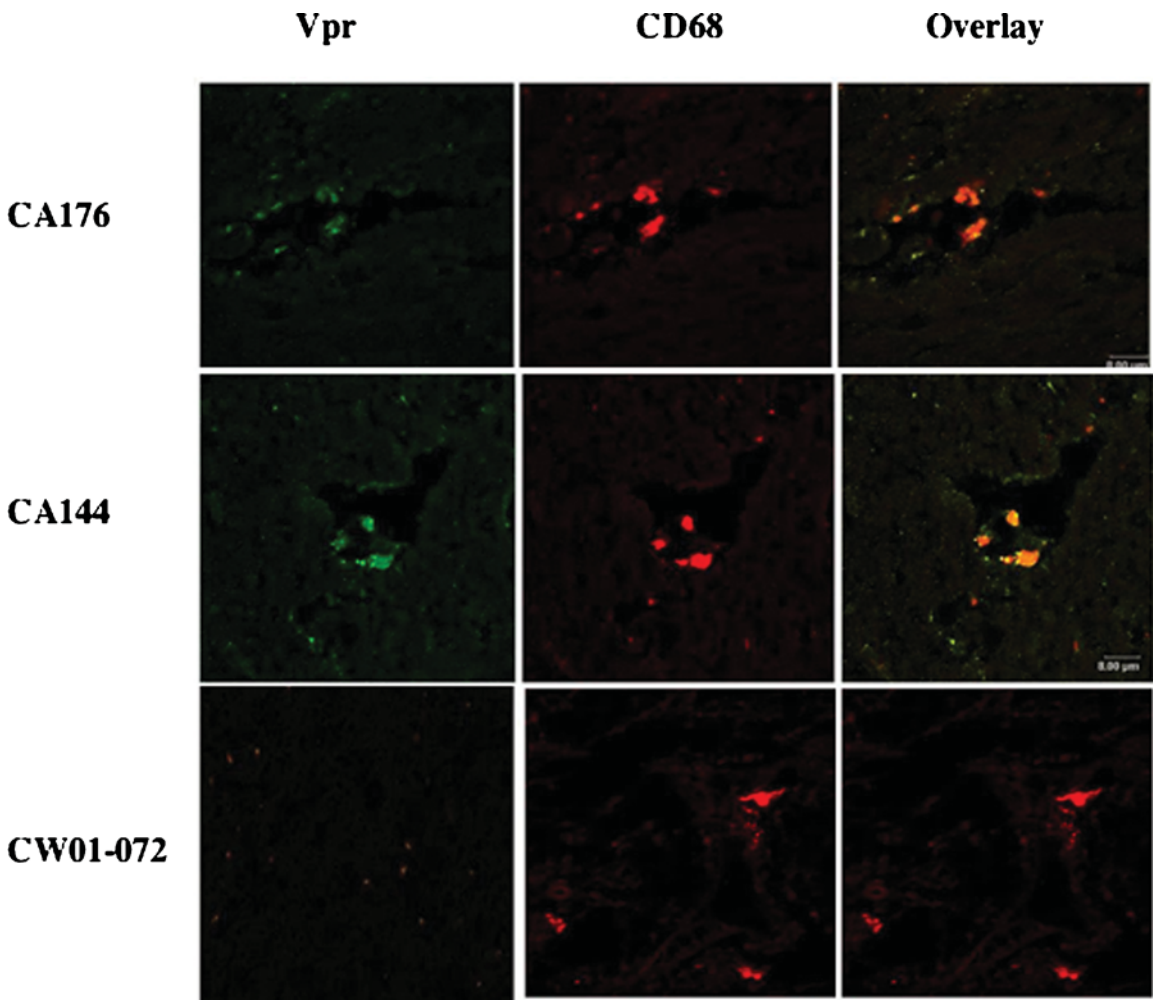

Figure 3 HIV-1 Vpr and CD68 double label immunofluorescence. Primary antibodies against Vpr (*green*) and CD68 (*red*) were used to show the presence of Vpr in the infiltrating and macrophages of patients CA176 and CA144. Colocalization of Vpr and CD68 is demonstrated by yellow in the overlay panel. CW01-072, the seronegative donor tissue sections stained for Vpr. Pictures were taken at 600× magnification using a laser confocal microscope. Macrophages are indicated with arrowhead.

positive cells. Similar Vpr-positive staining was also observed in patient samples CE144 and CA176 (data not shown). These results were surprising due to the fact that the neurons are not susceptible to HIV-1 infection. This could be possible given the hypothesis that Vpr produced in infected macrophages could be taken up by the neurons via transduction and/or other cellular uptake mechanism(s). Both seronegative controls and seropositive with immunoglobulin (IgG) controls did not show any positive staining for Vpr either in the frontal cortex or basal ganglia (data not shown).

Discussion

HIV-1–associated dementia (HAD) is the most severe neurological complication associated with HIV-1 infection. The neuronal damage and loss seen during HIVE has been primarily caused by apoptosis (Adle-Biassette *et al*, 1995; Gelberd *et al*, 1995). These neurotoxic effects are due in part to HIV-1 viral proteins either directly interacting with neurons or indirectly by releasing neurotoxins from the infected macrophages thus causing neuronal damage or death (Giulian *et al*, 1990; Garden, 2002). Several HIV-1 viral proteins including gp120, Tat, Nef, and Vpr are associated with the neuronal apoptosis seen in HIVE. Investigating further the ability of viral proteins to induce neurotoxic effects, results indicate that the CNS compartment is susceptible for virus infection and viral proteins are present *in vivo* (Hudson *et al*, 2000; Jones *et al*, 2000). Despite the suggested roles of Vpr in HIV neuropathogenesis, the presence of Vpr in HIVE brain tissue has not yet been demonstrated. Using a panel of HIVE patients for the first time we have shown the presence of Vpr *in vivo*.

Immunodetection results of HIV-1 p24 in brain tissue of the HIVE patients were in corroboration with previously published reports (Fischer-Smith *et al*, 2001). Furthermore, these results indicate active replication of HIV-1 virus in the brain macrophages

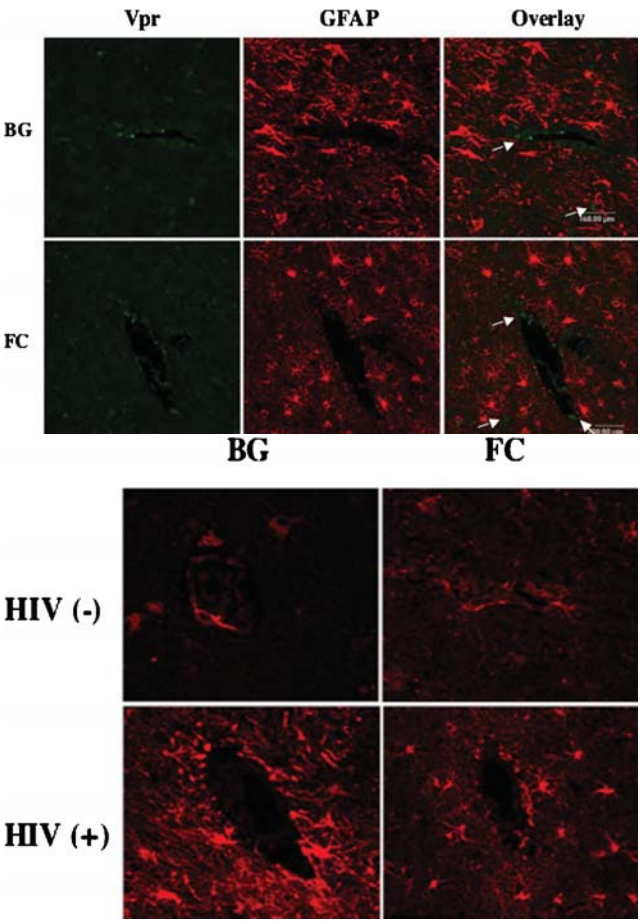

Figure 4 (**A**) HIV-1 Vpr and GFAP double-label immunofluorescence. Primary antibodies to Vpr (*green*) and GFAP (*red*) were used to determine if Vpr was present in the astrocytes of the HIVE brain tissue. No Vpr and GFAP colocalization was seen in either the basal ganglia (BG) or frontal cortex (FC) of this patient. Arrows indicate the Vpr positive macrophages. (**B**) Comparison of astrogliosis in HIV-1–negative and –positive patients. Increased staining of GFAP marker indicate the presence of a large number of astrocytes in HIV (+) samples compared to HIV (−) patients' samples. Pictures were taken at 600× magnification.

of the HIVE patients. Based on the p24 staining, it is possible to predict that HIV-1 viral replication could result in *de novo* synthesis of viral proteins in measurable quantities as well. Along these lines, we observed Vpr staining in HIV-1–positive brain macrophages. Although astrocytes have been shown to be susceptible to HIV infection, none of the HIVE tissues were positive for the presence of Vpr in astrocytes. This could be due to the sensitivity of the assay used to test for the presence of Vpr. The double-label immunofluorescence staining used to test the presence of Vpr in astrocytes is known to be less sensitive at detecting protein in tissue sections when compared to the avidin-biotin technique used for the single-label immunohistochemistry studies. Secondly, this could be due to the fact that though astrocytes are persistently infected, they may not support productive infection. This could also explain the presence of early proteins such as Nef present in astrocytes (Tornatore *et al*, 1994) and not late transcripts such as Vpr. This could be due to the restricted rev function in astrocytes as suggested by several studies (Fang *et al*, 2005; Gorry *et al*, 2003).

Interestingly, we also noticed that about 2% to 5% of neurons were positive for Vpr. Though neurons are neither infected by HIV-1 nor support virus replication, the presence of Vpr in neurons could be due to the result of free Vpr uptake by neurons. It has been reported that Vpr is present in multiple forms in the CSF of HIV patients *in vivo* (Levy *et al*, 1994; Tungaturthi *et al*, 2003). These forms include *de novo* synthesis of Vpr, Vpr associated with virus particles both infectious and noninfectious, and free Vpr (cell- and virus-free). Secondly, the transduction ability of Vpr to gain entrance into cells provides an attractive possibility for Vpr to affect cells in the CNS that are not generally infected by HIV-1. Showing the presence of Vpr in detectable amounts *in vivo* in the neurons of HIVE brain tissue further supports the neuropathogenic role of Vpr. Previous studies have shown that Vpr induced apoptosis in neurons through a caspase 8–dependent mechanism (Patel *et al*, 2000; Pomerantz, 2004). Together these studies provide ample *in vitro* evidence for the role of Vpr in the induction of neuronal apoptosis. Additionally several studies also suggest that axonal injury as the major neurological dysfunction in HIVE patients and there is a direct correlation has been observed in HIV-1 positive patients' brain tissue (Adle-Biassette *et al*, 1995; Goimetto *et al*, 1997) as well as one of the neuropathological outcome in several CNS diseases including AIDS (Medana and Esiri, 2003; Kennedy *et al*, 2004). Though *in vitro* studies have established a role for in Vpr in neuronal apoptosis, it is not known whether it is a consequence of axonal injury as well as the role of Vpr in axonal damage. Further studies assessing the role of HIV-1 Vpr in axonal injury are important and are in progress.

In conclusion, results presented here for the first time show that Vpr is present *in vivo* in detectable amounts in the brain tissue of HIVE patients. More specifically, Vpr was shown to be present in the macrophages and neurons, but not the astrocytes. By showing the presence of Vpr in HIVE brain tissue *in vivo*, we have come one step closer to understanding the role of Vpr in neuropathogenesis. Furthermore, by confirming that Vpr is present in the macrophages and neurons of HIVE brain tissue, we have established a link that could help further delineate Vpr-mediated neuropathogenesis and aid in the development of therapeutic treatments targeting the CNS compartment.

Materials and methods

Tissue specimens

Study population was selected based on their diagnosis of HIVE, and tissue specimens were obtained from

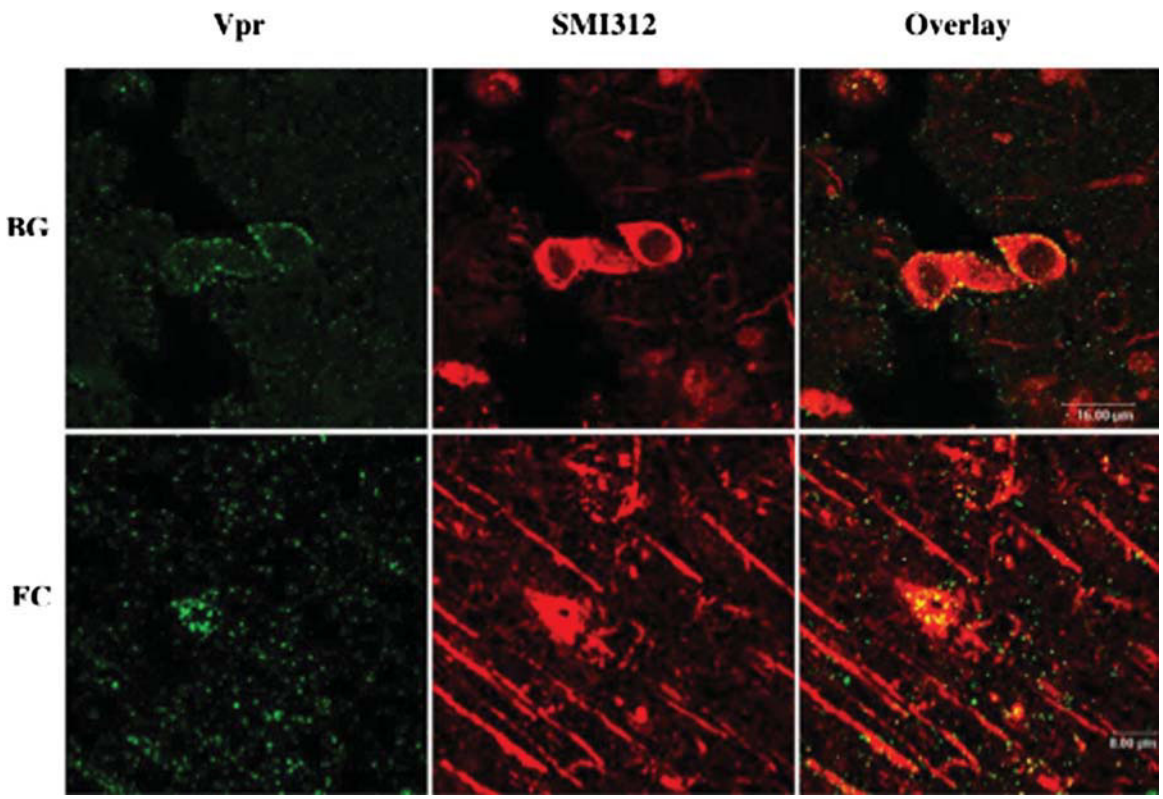

Figure 5 HIV-1 Vpr and neuronal marker, SMI312 double-label immunofluorescence. Primary antibodies to Vpr (*green*) and SMI312 (*red*) were used to determine the presence of Vpr in the neurons of the HIVE brain tissue. Colocalization of Vpr and SMI312 is visualized by yellow staining seen in the overlay column. Pictures were taken at 600× magnification.

the National Neurological AIDS Bank (University of California, Los Angeles), the California NeuroAIDS Tissue Network (University of California, San Diego), and the Manhattan HIV Brain Bank (Mount Sinai Medical Center) through the National NeuroAIDS Tissue Consortium (NNTC). Seronegative donor tissue sections from the basal ganglia and frontal cortex were received from the Tissue Bank of University of Pittsburgh, School of Medicine, as a kind gift from Dr. Ronald Hamilton. All samples were obtained using appropriate Internal Review Board (IRB) approval. Tissue sections received as formalin-fixed paraffin-embedded cassettes were then cut into 5-micron sections and floated onto a glass slide (Fisher Scientific, IL), followed by air drying at room temperature for 12 h. The slides were then stored at room temperature for future staining.

Immunohistochemistry for HIV-1 p24

Paraffin-embedded tissue sections were deparaffinized in Histoclear (National Diagnostics, GA) three times for 5 min each. The tissue sections were then rehydrated by shaking them manually through a series of descending alcohol concentrations (100% two times for 3 min, 95%, 75%, 50%, and 20% each for 2 min), followed by 2 min incubation in deionized H_2O (dH_2O). The slides were then treated for 20 min in 3% H_2O_2–MeOH at room temperature to block endogenous peroxidase activity, followed by another two min incubation in dH_2O. Antigen retrieval was performed by treating the slides with 0.4% pepsin (Dako Cytomation, CA) at 37°C for 10 min, followed by 5-min incubation at room temperature that allowed protein molecules to properly refold. The slides were washed with 1× phosphate-buffered saline (PBS) (Sigma, MO) for 10 min. In order to minimize nonspecific binding of antibodies, the tissue sections were then blocked in 10× Normal Donkey Serum (Jackson Immunoresearch Laboratories, PA) for 45 min at room temperature. After washing the slides three times for 5 min each in 1× PBS, the slides were exposed to the primary monoclonal mouse p24 antibody (1:10; Nova Castra, United Kingdom) for 12 h at room temperature. The slides were then washed three times for 5 min each in PBS/T (0.05% Tween 20) and exposed to the secondary biotin-SP–conjugated donkey anti-mouse antibody IgG (1:200; Jackson Immunoresearch Laboratories) for 30 min at room temperature. The tissue sections were then treated with Vectastain ABC kit (Vector Laboratories, CA) for 30 min at room temperature, followed by three 2-min washes with PBS/T. Antigen was visualized by incubating the slides with NovaRed substrate kit (Vector Laboratories, CA) for 5 min at room temperature. Tissue sections were counterstained with Mayer's Hematoxylin Solution

(Sigma-Aldrich, MO) and mounted with Aqueous Mount media (Scytek Laboratories, UT). The tissue sections were viewed and captured with bright field microscopy on a SPOT digital camera mounted on a Nikon E600 microscope (Nikon, NY). Images were processed using a Metaview software package (Universal Imaging, PA).

Immunohistochemistry for HIV-1 Vpr

Tissue sections were deparaffinized and rehydrated as described before. Several antigen retrieval techniques were used to unmask the Vpr epitopes. The first antigen retrieval solution tested was a citrate buffer (Dako Cytomation) at 96°C for both 20- and 40-min incubations. The second antigen retrieval solution tested was a 1 mM EDTA (pH 8.0) at 85°C for 30 min. The final antigen retrieval solution tested was the antigen retrieval treatment chosen for use in these experiments. This antigen retrieval treatment was 0.4% pepsin at 37°C for 20 min and then blocked in 10× Normal Donkey Serum for 45 min at room temperature. Sections were exposed to the primary antibody, a HIV-1 rabbit polyclonal Vpr-specific antibody (1:250; a kind gift from Dr. John Kappes, University of Alabama) for 12 h at 4°C in a humid chamber. Standardization of the primary antibody dilution was performed using 1:125, 1:500, and 1:1,000 dilutions, and the 1:250 dilution proved to produce the best staining and was used in these experiments. In addition, primary antibody incubation was tried at 37°C for 1 or 2 h, room temperature for 2 h or 12 h, and 4°C for 12 h, but the results shown in this study are the product of the incubation of the tissue sections with the primary antibody at 4°C for 12 h. The slides were then washed, and then exposed to the secondary biotin-SP–conjugated donkey anti-rabbit antibody IgG and the antigen was visualized using the NovaRed substrate kit as described above. Images were processed using a Metaview software package (Universal Imaging).

Double-label immunofluorescence

Deparaffinized and rehydrated sections were exposed to the primary antibodies (HIV-1 Vpr specific rabbit polyclonal antibody (1:100), and mouse monoclonal anti-GFAP (1:100; Dako Cytomation); mouse monoclonal anti-CD68 (1:25; Dako Cytomation); or mouse monoclonal anti-SMI 312 (1:200; Sternberger Monoclonals, MD) for 12 h at 4°C in a humid chamber. Following three 5-min washes in 1× PBS/T, the tissue sections were exposed to their respective secondary antibodies (goat anti-rabbit Cy2 1:200; Jackson Immunoresearch Laboratories); goat anti-mouse Cy3 (1:600; Jackson Immunoresearch Laboratories) for 2 h at 37°C. The antibodies were diluted in Normal Antibody Diluent (Scytek Laboratories). The slides were washed three times for 5-min each, and then glass cover slips were mounted using gelvatol (a kind gift from Wanda Wang, University of Pittsburgh School of Medicine, Neuropathology Department). The tissue was visualized using immunofluorescent laser confocal microscopy on a Leica-SL DMRE (Leica Microsystems, PA) at the Center for Biological Imaging (University of Pittsburgh, PA). Images were processed using Leica Confocal Software (Leica Microsystems).

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