DETECTION OF HIV-1 VIRAL PROTEIN R IN HIV ENCEPHALITIC BRAIN TISSUE

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Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2005

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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It was defended on

June 28, 2005

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HIV-1 Associated Dementia (HAD), the most severe neurological complication associated with HIV-1 infection, is commonly characterized by inflammation of the brain and neuronal degeneration, known as HIV Encephalitis (HIVE). HIVE develops in 20-30% of patients infected with HIV, which means that 9.5 million people are affected by HIVE throughout the world. While the introduction of highly active antiretroviral therapy (HAART) has decreased the incidence of severe late-stage HAD, the prevalence of its precursor HIVE is actually rising. Several HIV-1 viral proteins have been shown using *in vitro* models to have a role in the neurotoxic effects causing the neurodegeneration seen during HIVE. HIV-1 Viral Protein R (Vpr), a virion associated gene product which induces apoptosis in non-proliferating cells including neurons, is thought to contribute to the neuropathogenesis associated with HIVE. Previous studies have shown the presence of detectable levels of Vpr in the cerebrospinal fluid of HIV-1 infected patients. Extracellular Vpr released from HIV-1 infected macrophages has also been shown to be capable of transducing into cells not normally infected by HIV-1, causing death of these bystander cells. Additionally, Vpr has been shown *in vitro* to be able to induce apoptosis in human neurons. Although current research suggests that Vpr plays a significant role in neuropathogenesis, no work has been done yet *in vivo* to show the presence of Vpr in the brain

tissue of HIVE patients. Using a panel of eight HIVE and four HIV seronegative patient brain tissue sections, I performed immunohistochemistry staining for Vpr, p24, and brain cell specific markers. Results indicate that Vpr was present in detectable amounts in both the basal ganglia and frontal cortex of all eight HIVE brain tissue samples tested. Double label immunohistochemistry was performed using antibodies specific for astrocytes macrophages and neurons. I detected the presence of Vpr in the macrophages and neurons, but not in the astrocytes, of HIVE patients. The results of this study strongly support the role of Vpr in the neuropathogenesis seen during HIVE. Further studies based on these findings could lead to the development of effective therapeutic treatments necessary to reduce, and possibly prevent, this public health epidemic.

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ACKNOWLEDGEMENTS

First and foremost I would like to thank God for guiding me and protecting me through my journey here at GSPH.

I would like to thank Dr. Velpandi Ayyavoo for being my advisor throughout my stay at GSPH. Her knowledge and expertise in the field of science is an inspiration to us all. I would also like to thank my committee members Dr. Todd Reinhart and Dr. Cristian Achim for their guidance and advice towards this project.

Next I would like to thank the members of Dr Ayyavoo's lab. To Beth Schafer, thank you for being amazingly organized and understanding. To Michelle Janket, thank you for always listening to my daily frustrations in the lab, and helping me to figure out my troubleshooting options to resolve them. To Biswanath Majumder, thank you for the numerous engaging conversations about the effects of Vpr on apoptotic pathways. To Danielle McKeithen, Jayanth Venkatachari, Anamika Nath, and Nabanita Biswas, thank you for putting up with my overly anal control over the organization of the lab freezers.

I would also like to thank both my birth family and my church family. With their love, support, and prayers I was able to complete this project.

With deep appreciation, I would like to thank the large team of doctors, nurses, and lab technicians from Presbyterian University Hospital. Without their rapid diagnoses and efficient treatment I would not have been able to return to GSPH and finish this project.

Finally, I would like to thank my fiancé Duc Do. Without Duc lugging around my laptop, books, and papers I would not have been able to write this thesis. Thank you Duc for all your support and understanding these last few months, I love you.

1. INTRODUCTION

1.1. AIDS AND HIV-1

Acquired immunodeficiency syndrome (AIDS) was first reported in 1981 (1). It was not until 1985 that the causative agent of AIDS was identified and termed Human immunodeficiency virus type 1 (HIV-1) (2, 3). A recent report on the global AIDS epidemic, by the Joint United Programme on HIV/AIDS, indicated that 34.6-42.3 million people are infected with AIDS worldwide (4).

1.1.1. Genetic structure of HIV-1

HIV-1 is a lentivirus that belongs to the family *Retroviridae.* Common features found in all lentivirus infections include long and variable incubation periods, persistent viral replication, neurologic manifestations, and destruction of specific hematologic or immunologic cells (5). Like all lentiviruses, HIV-1 exhibits a tropism for macrophages, extensive genetic and antigenetic variability, and includes the presence of regulatory genes not found in other retroviruses (6).

HIV-1 encodes for structural, enzymatic, and accessory genes. The structural and enzymatic genes encoded by HIV-1 include *gag*, *pol*, and *env*. Gag serves as a precursor polyprotein that codes for the virion proteins matrix (MA), capsid (CA), and nucleocapsid (NC). MA is a HIV-1 virion protein involved in peripheral membrane stability. CA provides a shell

around the ribonucleoprotein complex, containing NC which binds to and protects the HIV-1 genomic RNA. Ribosomal frameshifting must occur for the production of the Gag-Pol precursor to be formed. The Pol precursor codes for additional virion genes, protease (PR), reverse transcriptase (RT), and integrase (IN). The main function of PR is its involvement in the proteolytic processing of the Gag-Pro-Pol precursors. RT performs three enzymatic activities involving RNA-dependent DNA polymerase, RNAase H, and DNA-dependent DNA polymerase, which mediate the transition of HIV-1 genomic RNA to viral DNA. The main role of IN is the integration of the HIV-1 viral DNA into the host chromosome. The Env precursor polyprotein encodes for the virion proteins surface (SU) and transmembrane (TM). SU and TM make up the virion surface receptor, more often referred to as gp160 (7).

1.1.2. HIV-1 regulatory and accessory proteins

HIV-1 encodes for two regulatory genes, Tat and Rev, and four accessory genes, Nef, Vpr, Vif and Vpu, in addition to the structural genes previously described. Tat, a HIV-1 regulatory protein, is a transcriptional transactivator. The main functions of Tat include transcriptional initiation, transcriptional elongation, and chromatin remodeling. At the host cellular level, Tat is known to induce apoptosis in $CD4^+$ T-cells and peripheral blood mononuclear cells (PBMCs) (8). Rev, also a HIV-1 regulatory protein, allows nuclear export of unspliced and singly spliced mRNAs encoding viral genes (9). Nef, a HIV-1 accessory protein, assists HIV-1 in evading the immune system, by downregulating the cell surface expression of MHC I (10), and by inducing the internalization and degradation of CD4 (11). Vpr is involved in the nuclear transport of the pre-integration complex (PIC), the induction of G_2 cell cycle arrest, and the transactivation of the HIV-1 LTR. Vpr has also been shown to both promote and

inhibit apoptosis (8). The main function of the HIV-1 accessory protein Vif is thought to be to ensure viral replication in non-permissive cell lines including $CD4^+$ T-cells and macrophages (12). Vpu also helps HIV-1 evade the immune system by downregulating the expression of cell surface CD4. In addition to this role, Vpu aids in the release of fully infectious virions, and induces apoptosis in $CD4^+$ T-cells (8).

1.2. THE CENTRAL NERVOUS SYSTEM : THE BRAIN

The central nervous system (CNS) is made up of the brain and the spinal cord. The main function of the CNS is to receive sensory stimuli from various parts of the body and to analyze this information. Once the information is analyzed, the CNS responds by generating signals that are transmitted throughout the body. The transmitted signals travel to peripheral nerves to initiate muscular, secretory, and other activities of the body (13).

1.2.1. Neurons

Neurons, the first major category of cells found in the brain, are directly responsible for conveying and processing any information in the brain. Their numerous microscopic processes extend from the neuronal cell body, or soma, in great distances throughout the brain and to other tissues (14).

Neurons are known to be polarized, which means that one part of the neuron is set to receive information, and another part is set to send information. The parts of the neuron responsible for the sending and receiving of the information are the axons and dendrites, respectively. Dendrites are shorter processes that extend from the neuronal cell body, and they are usually tapered and branched. The main function of the dendrites is to receive and integrate incoming information to the neuron. There can be several dendrites present on any one neuron. Axons are longer processes that can extend from the neuronal cell body in great distances. There is usually only one axon per neuronal cell body. The main function of an axon is to distribute signals from the neuron to other cells (14).

While processing signals, axons and dendrites of neurons work through specialized contacts known as synapses. These synapses serve as modes of communication between neurons, and from neurons to other cells and tissues. Electrical and chemical signals pass from a neuron through the synapse to a responding effector cell. Once the effector cell receives the signal it becomes activated.

There are four main classifications of neurons, and they differ based on the physical features of their axons and dendrites. Unipolar neurons are neurons that have an axon, but no dendrites. Pseudounipolar neurons contain one T-shaped branching axon, but no dendrites, and are commonly found in sensory ganglia. Bipolar neurons exhibit two separate axons which emerge directly and oppositely from the neuronal cell body. Multipolar neurons have one axon, and many dozens of dendrites, and are the most abundant neuron found in the CNS (14).

Neurons can also be classified based on their functional role in the brain. Motor neurons are those neurons involved in stimulating muscles or glands in the peripheral tissues. Sensory neurons on the other hand receive sensory stimuli from the environment or from the tissue and organs of the body. The most abundant neuron found in the brain though is the interneuron. The interneuron's function is to maintain connections and communication between the neurons in the CNS.

1.2.2. Brain Macrophages

Brain macrophages are the second main cell type found in the brain. The primary function of macrophages is to clear foreign substances from the tissue, including pathogens and other dead macrophages, through phagocytosis (15). Most brain macrophages are derived from circulating monocytes that have crossed the blood-brain barrier and entered the brain (16). Once

a monocyte enters the brain and sets up residence, it is then able to differentiate into a brain macrophage (17). Monocytes infiltrate into the brain to replace perivascular macrophages that become subject to injury by vascular problems or protein build up due to neurodegeneration (18). There are two main classes of brain macrophages, and they are distinguishable based on their location around the blood vessels of the brain (19). Infiltrating macrophages are those macrophages that have just entered the brain as monocytes and have gone through differentiation to become macrophages. Perivascular macrophages are the macrophages found lining the blood vessels of the brain. Brain macrophages are therefore the first line of defense against any pathogens that successfully enter the brain.

1.2.3. Glial Cells

1.2.3.1. Astrocytes

The glial cells are the third major category of cells found within the brain. Astrocytes are a particular kind of glial cell whose main function is to support neurons and make them more efficient and effective (20). Astrocytes support neurons by using their astrocytic processes involved with synapses to help neurons maintain their signaling capacity. Astrocytes also play an important role in brain homeostasis, by establishing contact with brain microvascular endothelial cells and regulating the permeability of the blood-brain barrier (18). The breakdown or increased permeability of the blood-brain barrier is an essential occurrence in the infiltration of any pathogen into the brain.

1.2.3.2. Oligodendrocytes

Another glial cell, known as an oligodendrocyte, is smaller then an astrocyte and has fewer processes. The small processes of an oligodendrocyte wrap around axons producing the myelin sheath that provides the electrical insulation of neurons in the CNS (14). Oligodendrocytes also produce the neurotrophic factors, including nerve growth factor (NGF) and neurotrophin-3, which are necessary for the development and survival of neurons.

1.2.3.3. Microglia

Microglia are the resident brain macrophages of the CNS (21). They have a dense ovoid or elongate nucleus, and minimal cytoplasm. Microglia are highly motile, and attracted to areas of brain injury. Once in those areas, microglia become highly phagocytic, and engulf and degrade the residues of damaged neurons and myelin. While performing there immunological function, microglia produce cytokines and chemokines which are toxic and can damage neurons and oligodendrocytes.

1.2.4. Basal Ganglia

The basal ganglia is the section of the brain responsible for movement. More specifically, the basal ganglia acts to facilitate voluntary movements, and to prevent competing movements that interfere with the bodies desired actions (13). The neuronal inputs into the basal ganglia are from the cerebral cortex and the frontal cortex of the brain. The neuronal outputs of the basal ganglia are directed mainly to the globus pallidus. Injury caused to the basal ganglia by disease results in unwanted competing motor patterns or difficulty in selecting motor pattern. Disease known to cause these disturbances in movement include Parkinson's disease and Huntington's disease (22).

1.2.5. Frontal Cortex

The frontal cortex is the section of the brain where higher levels of thinking, planning, and goal formulation take place (23). In particular, the planning that occurs in the frontal cortex is involved in appropriate behavioral responses to internal and external stimuli. The neuronal inputs into the frontal cortex come from the sensory and motor cortices, the thalamus, and the brain stem. The neuronal outputs from the frontal cortex are directed to the hippocampus, basal ganglia, cerebellum and thalamus. Injury to the frontal cortex brought on by disease results in impairment in planning, altered initiative, changes in personality, and reduced creativity (23). Alzheimer's disease has been shown to result in these impaired cognitive functions due to the injury that the frontal cortex sustains during the course of disease (24).

The frontal cortex contains mostly neurons, but brain macrophages and astrocytes are present as well. The basal ganglia, in contrast, contains mostly brain macrophages and astrocytes and a fewer number of neurons.

1.3. HIV-1 NEUROPATHOGENESIS

HIV-1 associated dementia (HAD) is the most severe neurological complication associated with HIV-1 infection (25). Pathological features leading up to HAD include inflammation of the brain and neurodegeneration, which is known as HIV encephalitis (HIVE) (26). Currently, 20-30% of people infected with HIV also exhibit HIVE (18). Since 34.6-42.3 million people are infected with HIV worldwide (4), and 20-30% of them are also exhibit HIVE, that translates into 9.5 million people living with HIVE worldwide.

1.3.1. How HIV-1 Enters the Brain

Circulating monocytes enter the brain and differentiate into infiltrating and perivascular macrophages (16). The circulating monocytes that enter the brain migrate there to replace those macrophages that have undergone injury from vascular problems and protein build up due to neurodegeneration (18). During this process of replenishing brain macrophages, the brain is exposed to intracellular pathogens which cross the blood-brain barrier inside the entering monocytes. This process is what is thought to be happening during the course of infection by HIV-1. Through what has become known as the "Trojan horse" hypothesis (27), HIV-1 is believed to ride into the CNS via the infected circulating monocytes. Once the infected monocyte has crossed the blood-brain barrier and entered the brain, it then differentiates into an infected brain macrophage. These brain macrophages are the cell type most commonly infected by HIV in the CNS (28). The infected infiltrating and perivascular macrophages can then produce a productive HIV infection within the CNS (29). HIV-1 virions can then be released

from the infected brain macrophages and be taken up by other macrophages or astrocytes within the brain parenchyma (30). Infected macrophages can also release HIV-1 viral proteins which can be taken up by bystander cells within the brain parenchyma (31-33). By using the "Trojan horse" mechanism, HIV has found a way to evade the host immune system and enter the brain to establish infection.

1.3.2. Pathological Manifestations of HIVE

During HIVE, a myriad of pathological manifestations can be found within the brain parenchyma. Since the basal ganglia and frontal cortex are the most predominant sites of HIV-1 infection during HIVE (34), the pathological features of these brain sections will be discussed in greatest detail here. The main features of HIVE in the CNS include wide spread reactive astrogliosis, myelin pallor, and infiltration of monocytes that differentiate into brain macrophages (35). Astrogliosis can be defined as an increase in the metabolic activity of astrocytes, and intense astrocytic proliferation. Astrogliosis can be found in the perivascular regions surrounding the blood vessels in the brain parenchyma of both the basal ganglia and frontal cortex. Myelin pallor can be seen as a pale region within the basal ganglia of the HIVE brain tissue. Myelin pallor results when ischemia induces the loss of myelin from around the axis cylinder of some nerve fibers.

Another pathological feature seen with HIVE, and often used in the diagnoses of HIVE, is the presence of multi-nucleated giant cells (36). Multi-nucleated giant cells are formed by the fusion of perivascular and parenchymal brain macrophages (37). During HIVE, multi-nucleated giant cells preferentially affect the basal ganglia (38). Additionally, microglial nodules, which

are created by an accumulation of resident brain macrophages known as microglia, are also observed in the basal ganglia and frontal cortex of HIVE patients (39).

In the presence of HIVE, infected macrophages in the CNS produce and release neurotoxins which can result in neuronal death (40). Neurons undergo dendritic pruning, simplification of synaptic contacts, and dramatic neuronal cell loss in the presence of neurotoxins (41). The damage caused to the neurons during HIVE results in the onset of cognitive and motor deficits (42). The most severe neuronal loss and damage is seen in the frontal cortex of HIVE patients (43, 44).

1.3.3. Effects of HAART on HIVE

Highly active antiretroviral therapy (HAART), which was created in 1995, is a treatment based on a strategy of using two or more drugs in combination to block different stages of HIV-1 viral replication (45). Since the introduction of HAART, there has been significant success in combating HIV related immune collapse and the former end stage complications of AIDS (46). HAART has also been shown to decrease the incidence of HAD (47). However, some studies have indicated that the prevalence of HIVE since the introduction of HAART is actually rising (48). The increase in the prevalence of HIVE is partially due to the increased survival rates of patients infected with HIV since the introduction of HAART (26). Another contributing factor to the increase in HIVE prevalence is based on the relative isolation of the CNS from systemic circulation. This isolation of the CNS allows latent or slowly replicating HIV-1 viruses residing in the CNS to be protected from the effects of HAART (18). Additionally, HAART is not available to 90 percent of the people infected with HIV due to cost concerns (49). Those people

who cannot receive HAART are also showing an increase in prevalence of HIVE. In summary, with or without the presence of HAART, the prevalence of HIVE is rising.

1.3.4. Role of HIV-1 Viral Proteins on Neurotoxic Effect

HIV-1 encodes several proteins that disturb cellular machinery of target cells within the CNS (50). The most common form of disturbance by the HIV-1 viral proteins on cells of the CNS is programmed cell death or apoptosis. HIV-1 viral proteins gp120 (51), Tat (52), Nef (53), and Vpr (54), are the viral proteins most often involved in the cellular disturbances in the CNS. During HIVE, gp120 interacts with chemokine receptors that are present on many cell types in the CNS (55). This interaction with gp120 alters the signaling pathways that govern neuronal growth (56), migration (57), and viability (58). *In vitro*, HIV-1 Tat protein produces deleterious effects on astrocytic and neuronal cells (59). Tat is released into the medium by infected cells (60), and taken up by uninfected bystander cells through endocytosis (61). Once taken up by astrocytes, HIV-1 Tat can induce cell death by transactivating cellular genes involved in apoptotic signaling (62). Extracellular Tat can also cause neuronal cell death by activating N-methyl-D-aspartate receptors on neurons, and by increasing tumor necrosis factor alpha (TNFα) secretion (63, 64). Nef possesses cellular membrane fusion properties which may allow Nef to cross the cellular membrane and enter neurons (65). Once inside a neuron, Nef can induce neuronal cell death by activation of caspases and free radical production (66). The neurotoxic effects of Vpr will be discussed in greater detail in the following sections. Together, HIV-1 viral proteins can cause a dramatic neurotoxic effect during HIVE.

1.4. VPR CHARACTERISTICS AND FUNCTIONS

1.4.1. Vpr: Characteristics

HIV-1 viral protein R (Vpr) is a 96 amino acid, 14 kDa protein (67). Vpr is the only non-structural accessory protein packaged in the progeny HIV-1 virion in detectable quantities (68). The packaging of approximately 200 copies of Vpr per HIV-1 virion occurs through its interaction with the p6 region of Gag (69). There are three available forms of Vpr present in an HIV infected individual. The three available forms are cell associated Vpr, virion associated Vpr, including infectious, infectious non-productive, and non-infectious defective viruses, and finally free Vpr, cell-free and virus-free (70). Extracellular Vpr, or free Vpr, can increase the cellular permissiveness to HIV-1 virus replication, and reactivate HIV-1 virus in latently infected cells (71).

1.4.2. Vpr: Functions

The main functions of Vpr that are known include the nuclear transport of the pre-integration complex, induction of G_2 cell cycle arrest, transactivation of the HIV-1 LTR, and both the induction and inhibition of apoptosis.

1.4.2.1. Nuclear transport of the pre-integration complex

Vpr can induce HIV-1 viral infection in non-dividing cells such as monocytes and macrophages (72). This infection of non-dividing cells is dependant upon entry of the HIV-1 viral pre-integration complex (PIC) into the cell nucleus. HIV-1 MA, IN, and Vpr are found inside the PIC, and are directly involved in nuclear targeting of the PIC (73). Vpr targets the proviral DNA

to the host cell nucleus early in HIV-1 infection through its interaction with the nuclear pore complex proteins, contributing to the disruption of the nuclear envelope (74, 75). This disruption of the nuclear envelope leads to the nuclear import of the proviral DNA (76).

1.4.2.2. G_2 Cell Cycle Arrest

Vpr induces G_2 cell cycle arrest in proliferating cells by two mechanisms. Both mechanisms involve the activity of Vpr suppressing the activation of mitotic CDK. The first mechanism of Vpr induced G_2 cell cycle arrest involves Vpr enhancing the nuclear import of the upstream regulator of cdc25, protein phosphatase 2A (PP2A) (77). PP2A then dephosphorylates cdc25, ultimately leading to G_2 cell cycle arrest. The second mechanism of Vpr induced G_2 cell cycle arrest is by Vpr activating the expression of the CDK inhibitor p21/Waf1/Cip1 (78). Free Vpr can also transduce through the cytoplasmic membrane of cells resulting in the G_2 cell cycle arrest of bystander cells. Prolonged G_2 cell cycle arrest will lead the host cell to death by apoptosis. The G_2 cell cycle arrest induced by Vpr allows for the additional time needed for HIV-1 protein synthesis and virion packaging (79).

1.4.2.3. Transactivation of the HIV-1 LTR

Vpr enhanced HIV-1 viral replication is a result of the transactivation of the HIV-1 LTR by Vpr. Vpr binds to the glucocorticoid response element (GRE) sequences present within the HIV-1 LTR, which enhances the transcriptional activity of the HIV-1 LTR (80). Vpr also interacts with p300, a co-activator that regulates NF-kappaB dependant transcription, and is essential for HIV-1 LTR activation (81). Therefore, Vpr promotes transcription of HIV-1 by connecting transcription components and co-activators to the HIV-1 LTR.

1.4.2.4. Vpr: Apoptosis

Vpr induces apoptosis prior to T-cell receptor mediated activation, and inhibits apoptosis following T-cell receptor apoptosis. By inducing the NF-kappaB inhibitor, IkappaB, Vpr is able to regulate apoptosis (82). Additionally, Vpr promotes the upregulation of Bcl-2 associated death promoter (Bad), as well as other pro-apoptotic genes in the mitochondrial pathway of apoptosis, leading the cell to death by apoptosis (83). Prolonged G_2 cell cycle arrest can also lead a cell to apoptosis (84).

In contrast, at the onset of HIV-1 infection, low levels of Vpr inhibit the host apoptotic response, prolonging HIV-1 viral replication (85). Vpr can also promote anti-apoptotic effects in bystander cells (86). This anti-apoptotic effect on bystander cells could lead to a pool of persistently infected cells, resulting in an increase in the spread of HIV-1 virus. Additionally, Vpr can upregulate bcl-2 oncogene, which exhibits an anti-apoptotic effect as well (87). Therefore, Vpr can both induce and inhibit apoptosis depending on the stage of HIV-1 infection.

1.5. VPR ROLE IN NEUROPATHOGENESIS

Vpr has been suggested to have a pertinent role in the contribution to neuropathogenesis seen in HIVE. Importantly to note, Vpr has been proven to be present in detectable amounts in the CSF of patients infected with HIV-1 (88). Extracellular Vpr can transduce through intact cytoplasmic membranes and enter into cells that are not normally infected by HIV-1, and cause the death of these bystander cells (72). This transduction property of Vpr is meaningful in the context of HIVE because neurons are not normally infected by HIV-1. Extracellular Vpr can also form ion channels across the planar lipid bilayers of the neuronal cellular membrane (89). These ion channels disturb the ionic gradient of the neuronal cells, causing a large inward cation current and depolarization of the plasmalemma, resulting in neuronal cell death. It is the amino terminal region of Vpr that forms the ion channels that induces the neurotoxic effect (90). Vpr can also induce apoptosis in human neurons through the activation of caspase 8 (54). This is significant because neuronal loss due to apoptosis of neurons is a hallmark feature of HIVE (43, 44). Additionally, Vpr upregulates the pro-apoptotic molecule Bcl-2 associated death promoter (Bad)in neuronal cells (83), thus leading to neuronal apoptosis. Collectively, Vpr can transduce into neurons, induce apoptosis, and cause neuronal cell death using various apoptotic pathways.

Despite the findings of previous studies suggesting that Vpr plays a significant role in the neuronal loss seen during HIVE, no *in vivo* studies have been performed to show the presence of Vpr in HIVE brain tissue. Therefore, the purpose of this body of work is to determine if Vpr is present in detectable amounts in the brain tissue of HIVE patients. Through the use of immunohistochemistry, I determined that Vpr is present in the macrophages and neurons, but not

the astrocytes, in the brain tissue of 8 HIVE patients. The findings of this study strongly support the potential role of Vpr in neuropathogenesis displayed during HIVE.

2. SPECIFIC AIMS

HIV-1 Associated Dementia (HAD) is the most common cause of dementia in HIV infected patients. HAD is characterized by neuronal inflammation and degeneration, also known as HIV Encephalitis (HIVE). HIV-1 Viral Protein R (Vpr) is a virion associated gene product which induces apoptosis in non-proliferating cells including neurons (90). Vpr is thought to play a role in contributing to HIVE neuropathogenesis (18). The rationale behind the role of Vpr in neuropathogenesis stems from the finding that Vpr is present in detectable amounts in the CSF and other body fluid of HIV infected patients (88). It has also been shown that Vpr, in its free form, is able to transduce into human astrocytes and neurons (72). In addition, Vpr has been shown to induce apoptosis in astrocytes and neurons, leading to neuronal loss and damage as seen in HIVE (83, 91). Vpr has not however been shown *in vivo* in the brain tissue of HIVE patients. Therefore, the overall aim of this project was to determine the presence of detectable amounts of Vpr in the cell types present in the brain tissue of HIVE patients.

Specific Aim #1: Determine the presence of detectable amount of Vpr in HIVE brain tissue.

Rationale: Vpr has been shown to be present in detectable amounts in the CSF of HIV infected individuals (88). Furthermore, Vpr has been shown to induce apoptosis in human astrocytes and neurons *in vitro* (83, 91). Despite the evidence from previous studies suggesting a role for Vpr in HIVE neuropathogenesis, Vpr has not yet been shown *in vivo* in HIVE brain tissue. Therefore, through immunohistochemistry techniques, I propose to confirm the presence of detectable amounts Vpr in the HIVE brain tissue.

Specific Aim #2: Determine the presence of detectable amounts of Vpr in those cell types present in the HIVE brain tissue. Rationale: The basal ganglia and frontal cortex brain sections contain three major cell types involved in HIVE (18). These cell types include brain macrophages, astrocytes and neurons (18). Perivascular macrophages and astrocytes are known to be susceptible to infection with HIV (28, 92). In addition, perivascular macrophages are also known to support productive HIV infection in the brain (29). Furthermore, neuronal degeneration and inflammation have been shown to be present during HIVE (93). Free Vpr released from macrophages has been shown *in vitro* to be capable of transducing into cells not normally infected by HIV, causing death to these bystander cells (72). *Vpr* has also been shown *in vitro* to be able to induce apoptosis of human astrocytes and neurons (83, 91). Despite the previous studies suggesting the presence of Vpr in macrophages, astrocytes and neurons during HIVE neurodegeneration, no *in vivo* data has been shown to support this. Therefore, through double label immunohistochemistry and immunofluorescent laser confocal microscopy, I propose to determine the presence or absence of Vpr in the macrophages, astrocytes, and neurons of the HIVE brain tissue.

3. MATERIALS AND METHODS

3.1. TISSUE SPECIMENS

Approval for use of the HIVE brain tissue sections was granted by the National NeuroAIDS Tissue Consortium (NNTC). Tissue sections from the basal ganglia and frontal cortex of eight HIVE patients were received from three of the NNTC facilities, including 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). Tissue sections from the basal ganglia and frontal cortex of four HIV seronegative patients were received from the University of Pittsburgh, School of Medicine, Neuropathology Tissue Bank (a kind gift from Dr. Ron Hamilton).

3.2. TISSUE PROCESSING

Tissue sections were received from the California NeuroAIDS Tissue Network (University of California, San Diego) on three HIVE patients (CA176, CE129, and CE144). The tissue sections were received as formalin-fixed paraffin embedded cassettes. Two cassettes were received for each of the three HIVE patients, representing the basal ganglia (caudate) and frontal cortex (precentral gyrus) of each HIVE patient. The tissue blocks were then cut into 75 adjacent tissue sections at 5 microns each using a microtome tissue cutter (Giangarlo Scientific Company,

Pittsburgh, PA). The tissue sections were individually floated briefly in a 45ºC water bath so that the tissue sections could then be floated onto a glass slide (Fisher Scientific, Hanover, IL). After the tissue sections were on the glass slides, the slides were allowed to air dry for 12 hours at room temperature. The tissue slides were then stored at room temperature until used for staining. A total of 450 glass slides representing the basal ganglia and frontal cortex of these three HIVE patients were created with this technique.

Tissue sections pre-cut to 5 microns and on glass slides were also obtained for use in this study. Two patients (10017, and mhbb500) were received from the Manhattan HIV Brain Bank (Mount Sinai Medical Center). Six slides from the basal ganglia (caudate) were received from each of the two patients. Three patients (ABBN-68-4041, NNAB 2035, and NNAB 2066) were received from the National Neurological AIDS Bank (University of California, San Diego). Six slides from the frontal cortex (precentral gyrus) were received from each of the three patients.

HIV seronegative tissue sections were obtained from University of Pittsburgh School of Medicine Neuropathology Tissue Bank. Four patients were used in the seronegative studies. Eight slides representing the basal ganglia (caudate) and frontal cortex (precentral gyrus) of each of the four HIV seronegative patients were received. The tissue sections were received as paraffin embedded tissue sections cut at 5 microns and air dried onto glass slides.

3.3. IMMUNOHISTOCHEMISTRY FOR p24

Paraffin embedded tissue sections were deparaffinized inside a fumehood in Histoclear (National Diagnostics, Atlanta, GA) three times for five minutes each. The tissue sections were then rehydrated by shaking them manually through a series of descending alcohol concentrations (100% two times for 3 minutes, 95%, 75%, 50% and 20% each for 2 minutes) followed by a 2 minute incubation in deionized H_2O (dH_2O). The slides were then treated for 20 minutes in $3\%H_2O_2$MeOH at room temperature to block endogenous peroxidase activity, followed by another 2 minute incubation in dH_2O. The slides were then treated for antigen retrieval by treating them with 0.4% pepsin (Dako Cytomation, Carpinteria, CA) for 10 minutes at 37ºC. Antigen retrieval was necessary because the tissues were formalin fixed when processed. Most epitopes are masked but not destroyed when tissue sections are formalin fixed. The 0.4% pepsin antigen retrieval treatment allows for the unmasking of the epitopes in the formalin fixed tissues, so that they can be recognized by antibodies. After a 5 minute incubation at room temperature to allow protein molecules to properly refold, the slides were washed with 1x phosphate buffered saline (PBS) (Sigma, St. Louis, MO) for 10 minutes. The tissue sections were then circled with an ImmEdge hydrophobic pen (Vector Laboratories, Burlingame, CA) in preparation for further incubations. In order to minimize nonspecific binding of antibodies, the tissue sections were then blocked in 10x Normal Donkey Serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 45 minutes at room temperature. After washing the slides three times for 5 minutes each in 1x PBS, the slides were exposed to the primary monoclonal mouse p24 antibody (1:10; Nova Castra, Newcastle upon Tyne, United Kingdom) for 12 hours at room temperature.

The slides were then washed three times for 5 minutes each in PBS/T (0.05% Tween 20 (Fisher Scientific, Hanover Park, IL) in 1x PBS), and then exposed to the secondary Biotin-SP-conjugated Donkey anti-Mouse antibody IgG (1:200; Jackson Immunoresearch Laboratories, West Grove, PA) for 30 minutes at room temperature. Both the primary anti-p24 and the secondary donkey anti-mouse antibody were diluted in Normal Antibody Diluent (Scytek Laboratories, Logan, UT). The tissue sections were then treated with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by three 2 minute washes with PBS/T. The Vectastain ABC kit supplies avidin to bind to the biotin found conjugated to the secondary antibody. The avidin:biotinylated enzyme complex (ABC) formed is essentially irreversible due to the high affinity of avidin for biotin. Biotinylated peroxidase is also found bound to the avidin in the Vectastain ABC kit. The antigen was visualized using the NovaRed substrate kit (Vector Laboratories, Burlingame, CA) for 5 minutes at room temperature. The biotinylated peroxidase bound to avidin will interact with the NovaRed substrate, degrading the substrate and producing a colored reaction product. The colored reaction precipitate in the case of NovaRed is red, allowing the p24 antigen to be visualized red. After the slides were washed two times quickly in dH_2O, the tissue sections were counterstained with Mayer's Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO) for 3 minutes at room temperature. Following two quick washes in dH_2O, glass cover slips were mounted onto the slides with Aqueous Mount (Scytek Laboratories, Logan, UT). The tissue sections were viewed and captured with bright field microscopy on a SPOT digital camera mounted on a Nikon E600 microscope (Nikon, Melville, NY). Images were processed using a Metaview software package (Universal Imaging, West Chester, PA). Use of the Nikon E600 microscope and Metaview software package was kindly granted by Dr. Todd Reinhart.

3.4. IMMUNOHISTOCHEMISTRY FOR VPR

Paraffin embedded tissue sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) three times for five minutes each. The tissue sections were then rehydrated through a series of descending alcohol concentrations (100% two times for 3 minutes, 95%, 75%, 50% and 20% each for 2 minutes) followed by a 2 minute incubation in dH_2O. The slides were then treated for 20 minutes in $3\%H_2O_2MeOH$ at room temperature, followed by another 2 minute incubation in dH_2O.

Several antigen retrieval techniques were used to unmask the Vpr epitopes. The first antigen retrieval solution tested was a citrate buffer (Dako Cytomation, Carpinteria, CA) at 96ºC for both 20 and 40 minute incubations. The second antigen retrieval solution tested was a 1mM EDTA (pH 8.0) at 85ºC for 30 minutes. 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 37ºC for 20 minutes.

After a 5 minute room temperature incubation to allow protein molecules to properly refold, the slides were washed with 1x PBS (Sigma, St. Louis, MO) for 10 minutes. The tissue sections were then circled with an ImmEdge hydrophobic pen (Vector Laboratories, Burlingame, CA) in preparation for further incubations. The tissue sections were then blocked in 10x Normal Donkey Serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 45 minutes at room temperature.

After washing the slides three times for 5 minutes each in 1x PBS, the slides 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 hours at 4ºC 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, the incubation with the primary antibody was tried at 37ºC for 1 or 2 hours, room temperature for 2 hours or 12 hours, and 4ºC for 12 hours, but the results shown in this study are the product of the incubation of the tissue sections with the primary antibody at 4ºC for 12 hours.

The slides were then washed three times for 5 minutes each in PBS/T (0.05% Tween-20 (Fisher Scientific, Hanover Park, IL) with 1x PBS), and then exposed to the secondary Biotin-SP-conjugated Donkey anti-Rabbit antibody IgG (1:200; Jackson Immunoresearch Laboratories, West Grove, PA) for 30 minutes at room temperature. Additional dilutions tried with the secondary antibody were 1:1,000 and 1:500, but the results displayed in this study are with a 1:200 dilution of the secondary antibody. Additionally, the secondary antibody dilution was also tested at 37ºC for 30 minutes, but the incubation used for these studies was at room temperature for 30 minutes. Both the primary anti-Vpr and secondary donkey anti-rabbit antibodies were diluted in Normal Antibody Diluent (Scytek Laboratories, Logan, UT).

Following exposures to the primary and secondary antibodies, the tissue sections were treated with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by three 2 minute washes with PBS/T. The Vpr protein was visualized using the NovaRed substrate kit (Vector Laboratories, Burlingame, CA) for 10 minutes at room temperature. After the slides were washed two times quickly in dH_2O, the tissue sections were counterstained with Mayer's Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO) for 3 minutes at room temperature. Following two quick washes in dH_2O, glass cover slips were

mounted onto the slides with Aqueous Mount (Scytek Laboratories, Logan, UT). The tissue sections were viewed with bright field microscopy on a SPOT digital camera mounted on a Nikon E600 microscope (Nikon, Melville, NY). Images were processed using a Metaview software package (Universal Imaging, West Chester, PA).

3.5. DOUBLE LABEL IMMUNOHISTOCHEMISTRY

Paraffin embedded tissue sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) three times for five minutes each. The tissue sections were then rehydrated through a series of descending alcohol concentrations (100% two times for 3 minutes, 95%, 75%, 50% and 20% each for 2 minutes) followed by a 2 minute incubation in dH_2O. The slides were then treated for antigen retrieval with 0.4% pepsin (Dako Cytomation, Carpinteria, CA) for 20 minutes at 37ºC. After a 5 minute room temperature incubation, the slides were washed with 1x PBS (Sigma, St. Louis, MO) for 10 minutes. The tissue sections were then circled with an ImmEdge hydrophobic pen (Vector Laboratories, Burlingame, CA) in preparation for further incubations.

The tissue sections were then blocked in 10x Normal Goat Serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 45 minutes at room temperature. After washing the slides three times for 5 minutes each in 1x PBS, the slides were exposed to the primary antibodies (HIV-1 Vpr specific rabbit polyclonal antibody (1:100), mouse monoclonal anti-GFAP (1:100; Dako Cytomation, Carpinteria, CA); mouse monoclonal anti-CD68 (1:25; Dako Cytomation, Carpinteria, CA); mouse monoclonal anti-SMI 312 (1:200; Sternberger Monoclonals, Lutherville, MD) for 12 hours at 4ºC in a humid chamber. Following three 5

minute washes in PBS/T, the tissue sections were exposed to their respective secondary antibodies (goat anti-rabbit Cy2 (1:200; Jackson Immunoresearch Laboratories, West Grove, PA); goat anti-mouse Cy3 (1:600; Jackson Immunoresearch Laboratories, West Grove, PA) for 2 hours at 37ºC. The antibodies were diluted in Normal Antibody Diluent (Scytek Laboratories, Logan, UT).

The slides were washed three times for 5 minutes each, and then glass coverslips 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, Exton, PA) at the Center for Biological Imaging (University of Pittsburgh, Pittsburgh, PA). Images were processed using Leica Confocal Software (Leica Microsystems, Exton, PA).

4. RESULTS

4.1. DETECTION OF VPR IN HIVE BRAIN TISSUE

HIV-1 is thought to enter the brain via infected monocytes that cross the blood-brain barrier and differentiate into brain macrophages (27). Several HIV-1 viral proteins have been shown to be present in the CNS of patients infected with HIV-1, including gp120 (58) and Tat (94). Vpr has also been shown to be present in detectable amounts in the CSF of those patients infected with HIV (71). Additionally, Vpr has been shown to induce neuronal death through both apoptotic and necrotic mechanisms (83, 90). While several studies have been done that suggest a role for Vpr in neuropathogenesis, all of these results have been attained *in vitro,* and Vpr has not yet been shown *in vivo* in the brain tissue of HIV-1 infected patients. Therefore, I propose to determine if Vpr is present in detectable amounts in the brain tissue of HIVE patients.

4.1.1. Creating Patient Panel

In order to carry out the specific aims as described, a panel of brain tissue sections representing HIVE patients and HIV seronegative controls was required. In order to create this panel, brain tissue from the frontal cortex and basal ganglia were obtained from eight HIVE patients, ranging in age from 33 to 47 years, who had died of AIDS. These eight patients were chosen based on their diagnosis of HIVE, and tissue specimens were obtained from 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 permission by the National NeuroAIDS Tissue Consortium. Briefly, paraffin embedded HIVE brain tissues were sectioned at 5 microns, and affixed to glass slides. Approximately 450 slides representing the HIVE patients were created for use in this study. The patient demographics are summarized in Table 1.

Table 1: Patient Demographics

8 HIVE patients and 4 HIV seronegative patients were chosen to make up the patient panel. A variety of patients representing high and low viral load carriers, as well as HAART treated and HAART naïve were chosen.

Patient ID	HIVE status	Plasma Vir Ld	CSF Vir Ld	Age/Gender	HAART
CA176	HIVE	8	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	N/A	44M	Yes
NNAB 2066	HIVE	250000	N/A	34M	Yes
10017	HIVE	389120	>750000	44M	No
mhbb 500	HIVE	210000	N/A	47M	No
CW01-072	Seronegative	N/A	N/A	57M	N/A
CW01-073	Seronegative	N/A	N/A	48M	N/A
CW00-148	Seronegative	N/A	N/A	62M	N/A
CW99-100	Seronegative	N/A	N/A	54M	N/A

The basal ganglia and frontal cortex of these HIVE patients were chosen for this study based on previous HIVE pathological findings observed in these brain sections. During the course of HIV infection, the basal ganglia and frontal cortex are the predominant sites of productive HIV infection (34). Microscopically, the basal ganglia has been shown to contain multinucleated giant cells, a characteristic seen during HIVE (38). The frontal cortex has been shown to incur significant neuronal loss and damage in HIVE, resulting in a reduction in total neuronal number (43, 44).

HIV seronegative patient samples obtained from the University of Pittsburgh School of Medicine (Pittsburgh, PA) were used as negative controls in this panel. The seronegative control group, ranging in age from 48 to 62 years, had died from a variety of systemic illnesses.

Since the introduction of HAART, a decrease in the incidence of HAD has been seen, but the prevalence of HIVE is actually rising. To represent both the pre-HAART and post-HAART era, three patients who had never received HAART, and five patients who were at the time of death receiving HAART, were chosen for this study. A report by Chang *et. al.* (95) suggested that an increased plasma viral load may lead to an increase in astroglial proliferation, a characteristic seen during HIVE. To represent both high and low viral load carriers, eight patients with a broad range of plasma and CSF viral loads were chosen for this study.

4.1.2. Detection of p24 in HIVE Brain Tissue

HIV-1 capsid protein p24 has been shown to be the most abundant antigen present during HIV infection (96). Additionally, HIV-1 p24 has been shown to be present in HIVE brain tissue (97). Therefore, since p24 is present in greater amounts than Vpr during HIV-1 infection, I wanted to first show the presence of p24 in the HIVE patient samples as a positive control before I tested the samples for the presence of Vpr.

The patient samples attained and processed in Table 1 were stained for p24 antigen as a marker of HIV-1 infection. To determine if p24 was present in detectable levels in these HIVE patient samples, immunohistochemistry staining was performed on the 8 HIVE patient samples and the 4 HIV seronegative control samples. A primary mouse monoclonal anti-p24 antibody was used which recognizes the HIV-1 capsid protein p24. The secondary antibody used was a donkey anti-mouse biotinylated antibody. The secondary antibody provided the biotin necessary

for the avidin, made available from the Vectastain ABC kit, to bind to. The protein was then visualized using a NovaRed substrate kit which binds to the avidin:biotin complex making the proteins detected appear red. The nuclei of the cells present in the brain tissue were counterstained blue with Mayer's Hematoxylin Solution. The results of the p24 staining of the basal ganglia and frontal cortex of the HIVE patients and HIV seronegative controls can be seen in Figure 1.

As shown in Figure 1A and 1B, HIV-1 capsid protein was detected in the basal ganglia of the HIVE patients. Figure 1E and 1F display similar staining patterns showing the presence of p24 in macrophages (Figure 1E) and microglia (Figure 1F inlay) of the frontal cortex of the HIVE patients. The detection of p24 in the cytoplasm of the perivascular macrophages is indicative of HIV-1 replication in these cells. No p24 staining was seen in the basal ganglia or frontal cortex of the HIV seronegative controls (Figure 1C and 1G). In order to remove the possibility of the positive staining being due to the secondary antibody sticking non-specifically to the tissue, a control was used in which no primary antibody was added to the tissue section but the protocol was followed as described above (Figure 1D and 1H). Results indicated that no non-specific staining was occurring with the secondary antibody, which further confirms the validity of the p24 staining seen in Figure 1.

Collectively these observations indicate the presence of HIV-1 capsid protein in both the basal ganglia and frontal cortex of HIVE patients. These results are in agreement with previous studies showing the presence of p24 in the brain tissue of HIVE patients (97). Additionally, these results indicate that HIV-1 viral replication is occurring in the perivascular macrophages. This 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 a productive HIV-1 infection (28, 29).

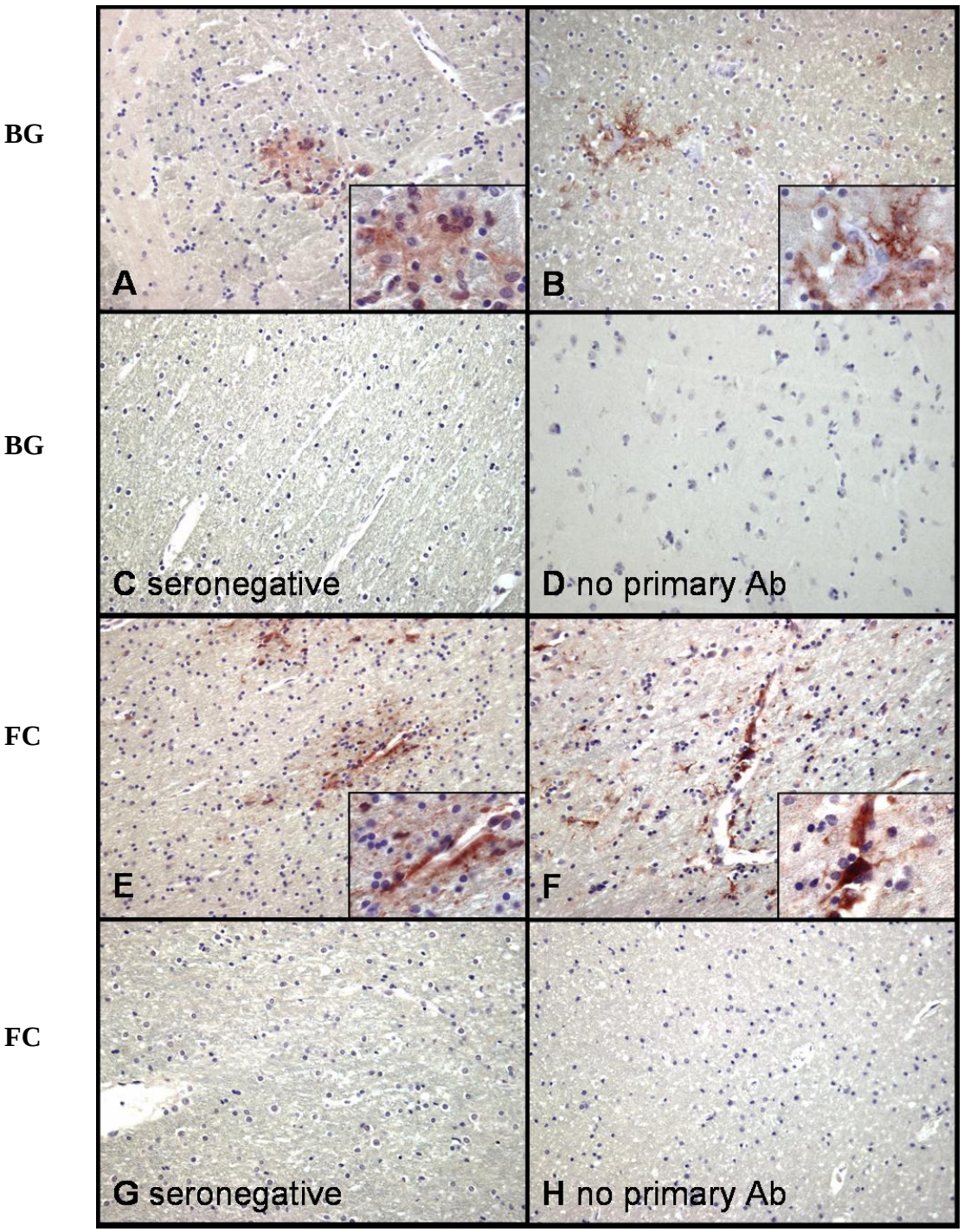

Figure 1: Detection of HIV-1 p24 antigen by Immunohistochemistry

(**A and B**) Detection of p24 in the basal ganglia of HIVE brain tissue (representative of 8 HIVE patients). (**E and F**) Detection of p24 in the frontal cortex of HIVE brain tissue (representative of 8 HIVE patients). (**C and G**) HIV Seronegative controls of the basal ganglia (BG) and frontal cortex (FC) respectively. (**D and H**) No primary antibody controls of the basal ganglia and frontal cortex respectively. Pictures were taken at 200x and inlays at 600x magnifications. p24 staining was visualized with NovaRed peroxidase substrate (red), and the nuclei were counterstained with Mayer's Hematoxylin Solution (blue).

4.1.3. Vpr Detection in HIVE Brain Tissue

HIV-1 Vpr is present in the HIV-1 infected host in three forms, Vpr in virus infected cells due to *de novo* synthesis, virion incorporated Vpr, and free Vpr (cell and virus free). Vpr is incorporated into the HIV-1 virion in significant amounts through its interaction with the p6 region of Gag (98-100). Additionally, the virion incorporated Vpr is present in both infectious and non-infectious virions, presenting a significant source of Vpr available to the host cells. Free Vpr protein has been shown to be able to transduce through intact cytoplasmic membranes into cells that are not normally infected by HIV-1, and cause death of these bystander cells (72).

The apoptotic properties of Vpr in both primary cells and many cell lines have been well established (101-103). In terms of the specific cells present in the brain, free Vpr has also been shown to induce apoptosis of human neuronal precursor cells, as well as mature differentiated neurons, through a caspase 8 dependent mechanism (54, 83). In addition, Vpr, through its amino-terminal region, has been shown to form cation-selective ion channels in planar lipid bilayers, disrupting the ionic gradient and causing a large inward current and cell death in neurons (89, 90). Vpr can also cause apoptotic and nectrotic cell death in astrocytes (91). Vpr has been shown to be present in detectable amounts in the CSF of individuals infected with HIV (88). Therefore, if Vpr does play a role in neuropathogenesis as suggested by previous studies, then Vpr should be detectable in the brain tissue of HIVE patients.

4.1.3.1. Optimization of Vpr Immunohistochemistry.

Despite the evidence from previous studies suggesting the role of Vpr in neuropathogenesis, Vpr has not yet been shown *in vivo* in the brain tissue of HIVE patients. Therefore it was necessary to optimize the conditions by which the tissue was treated in order to obtain accurate Vpr staining. 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) and keeping four points constant while modifying the fifth, I was able to develop a protocol that resulted in clear and consistent Vpr staining.

The antigen retrieval solution was the first step of immunohistochemistry that was optimized. Formalin-fixation and paraffin embedding can hinder or mask the immunoreactivity of certain proteins for immunohistochemical detection. Antigen retrieval techniques are used to de-mask the antigen so that it can be recognized for use in immunohistochemistry staining. When testing a new antigen that has never been detected by immunohistochemistry before, a battery of antigen retrieval techniques should be tried, because different antigens require different conditions for retrieval (104, 105). The three methods used in this study were derived from techniques previously found to work in similar immunohistochemistry protocols, and included a citrate buffer at 96ºC (106), 1mM EDTA (pH 8.0) at 85ºC (107), and 0.4% pepsin at 37ºC (108). The citrate buffer was used at 96ºC for both 20 and 40 minute incubations, but the tissue sections tested showed no increased levels of Vpr expression using this buffer when compared to tissue sections that were stained but not treated for antigen retrieval. In addition, the tissue sections were lifted off of the slides during the incubation due to the increased temperature and stringency of this method. EDTA (1mM, pH 8.0) was also tried at 85ºC for 30 minutes (Figure 2A). While the tissue sections remained intact and on the slides, no prominent Vpr staining was seen with the 1mM EDTA. The final antigen retrieval solution tried was 0.4% pepsin at 37ºC for 20 minutes. The 0.4% pepsin treatment resulted in vivid Vpr staining as seen in Figure 2B.

The primary antibody used in the detection of Vpr was a rabbit polyclonal Vpr-specific serum antibody (a kind gift from Dr. John Kappes, University of Alabama). There is no commercial antibody available for the detection of Vpr, but this Vpr specific antibody has been tested and found to be specific for Vpr in Western Blotting and immunoprecipitation techniques (109). The temperature and time combinations tested were 37ºC for 1 or 2 hours, room temperature for 2 hours or 12 hours, and 4ºC for 12 hours. The 4ºC for 12 hours incubation revealed the best staining.

This Vpr-specific primary antibody is routinely used in immunofluorescence (*in vitro* cell culture) analysis in our lab at a 1:250 dilution. I therefore wanted to try both higher and lower dilutions to determine the detectable threshold of the primary antibody during immunohistochemistry. The dilutions used were 1:1,000, 1:500, 1:250, and 1:125. The 1:1,000 and 1:500 dilutions revealed no Vpr staining. In contrast, the 1:125 dilution expressed high background levels (Figure 2C), masking the true Vpr staining. The 1:250 dilution produced clear protein expression with little to no background (Figure 2D).

The secondary antibody used was a biotinylated donkey anti-rabbit antibody. The manufacturers instructions for the secondary antibody suggested using a 1:200-1:1,000 dilution, I therefore tried a range of secondary antibody dilutions including 1:1,000, 1:500, and a 1:200. All three dilutions were incubated at both 37ºC for 30 minutes, or room temperature for 30 minutes. The secondary antibody produced the best staining results at a 1:200 dilution for 30 minutes at room temperature (Figure 2F). The 1:1,000 dilution exhibited no staining at either incubation condition, and the 1:500 dilution (Figure 2E) expressed only minimal staining at the 37ºC for 30 minutes incubation.

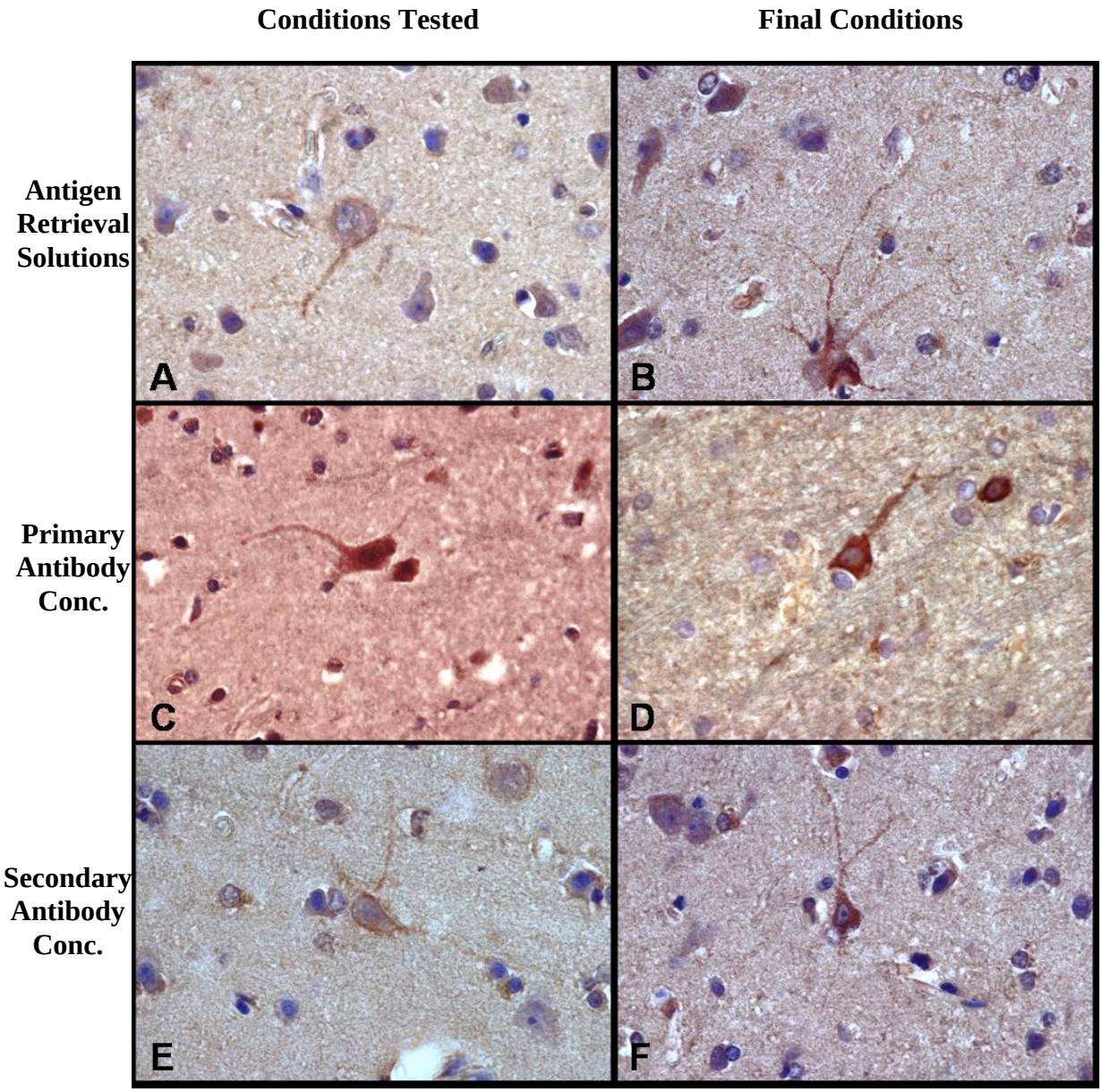

Figure 2: Optimization of Vpr Immunohistochemistry

The EDTA (1mM, pH 8.0) antigen retrieval solution tested (**A**) displayed less vivid staining for Vpr when compared to the 0.4% pepsin solution (**B**) used in the final Vpr immunohistochemistry protocol. The primary rabbit polyclonal Vpr antibody was tested at 1:1,000, 1:500, 1:250 (**D**), and 1:125 (**C**). High background levels were produced using the 1:125 dilution of the primary Vpr antibody (**C**) when compared to the 1:250 dilution of the primary Vpr antibody (**D**) used in the final Vpr immunohistochemistry protocol. The secondary biotinylated donkey anti-rabbit antibody at a 1:500 dilution (**E**) produced minimal Vpr staining when compared to the 1:200 dilution of the secondary antibody (**F**) used in the final Vpr immunohistochemistry protocol. Pictures were taken at 600x magnification.

NovaRed substrate (Vector Laboratories, Burlingame, CA), which through a proprietary chemical substrate reaction allows the protein to be visualized red, and DAB-Ni substrate (Vector Laboratories, Burlingame, CA), which allows the protein to be visualized black, were tested on the HIVE tissue sections at 5, 10, and 15 minute incubations. The NovaRed and DAB-Ni substrates produced similar staining intensities. Therefore, due to the carcinogenic properties of DAB-Ni, I chose to use NovaRed for future staining. The NovaRed substrate worked the best at a 10 minute exposure to the HIVE brain tissue.

In summary, the tissue sections were deparaffinized with Histoclear (National Diagnostics, Atlanta, GA), and rehydrated through a descending gradient of ethanol. The tissue sections were then treated with 0.4% pepsin for antigen retrieval at 37ºC for 20 minutes. The tissue sections were then exposed to the primary Vpr specific antibody for 12 hours at 4ºC in a humid chamber. The humid chamber allows the slides to be able to be incubated with the antibody for the desired 12 hours without drying out. The biotinylated secondary antibody was then used for 30 minutes at room temperature, followed by exposure to avidin. The protein was then visualized using the NovaRed substrate for 10 minutes.

4.1.3.2. Presence of Vpr in HIVE Brain Tissue

Using the optimized protocol described in section 4.1.3.1, all eight HIVE and four HIV seronegative patients were stained for Vpr by immunohistochemistry. Results of the staining in three HIVE patients are presented in Figure 3, and HIV seronegative controls in Figure 4. The patients shown in Figure 3 are presented based on the availability of both the basal ganglia and frontal cortex tissue for these patients. The staining presented in Figure 3 and 4 is a representative of that seen in all eight HIVE and four HIV seronegative patients tested.

As shown in Figure 3A, 3C, and 3E, Vpr was detected in the basal ganglia of the HIVE patients. Figure 3B, 3D, and 3F show representatives of the detection of Vpr in the frontal cortex of the HIVE patients. While only three representatives are shown for the basal ganglia and frontal cortex, Vpr staining was present in all 8 HIVE patients. This is an important observation because Vpr has never been shown in the brain tissue before this study.

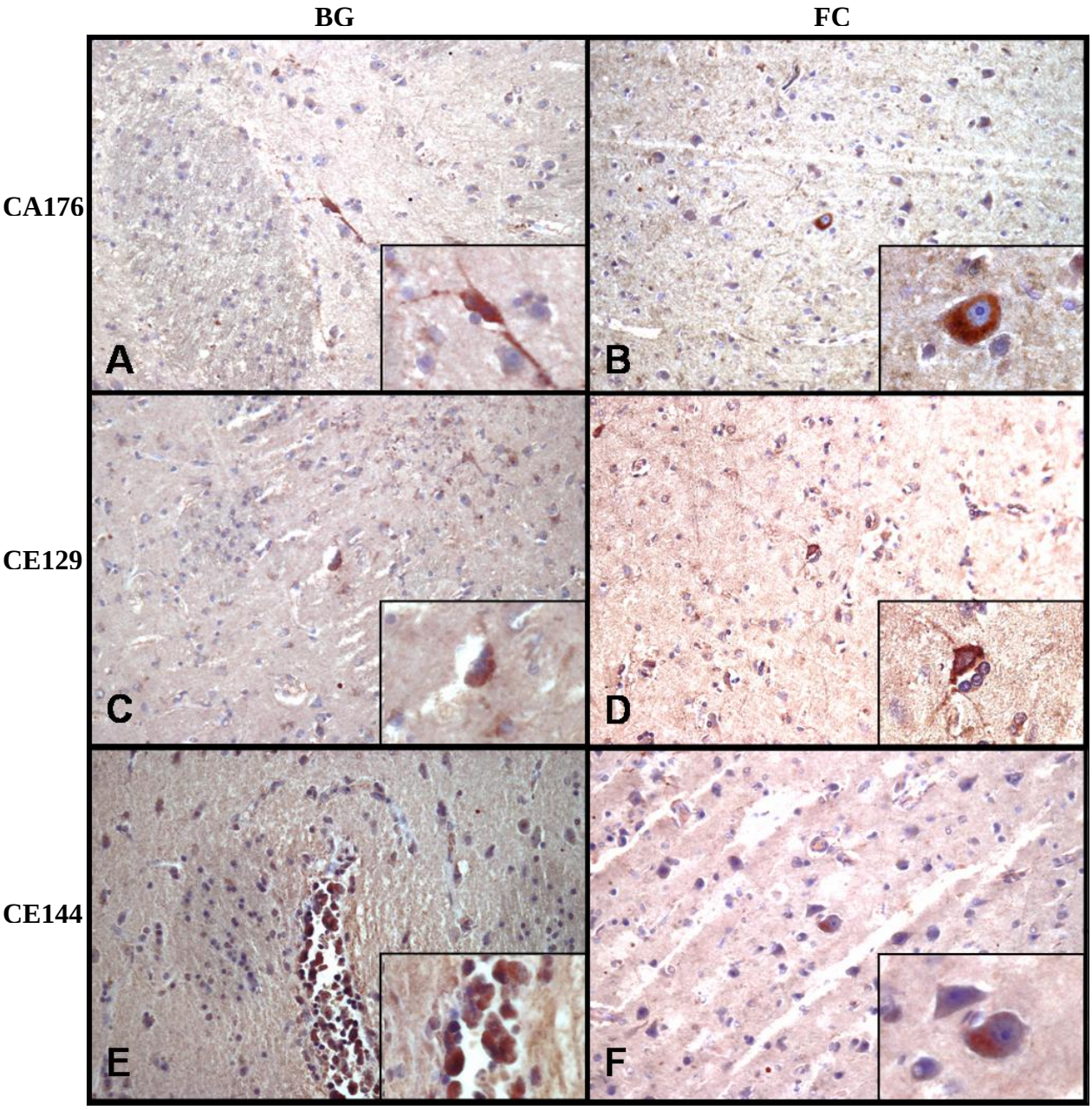

Figure 3: Detection of HIV-1 Vpr by Immunohistochemistry

Positive Vpr staining was seen in the basal ganglia (**A,C, and E**) and in the frontal cortex (**B, D, and F**). Pictures were taken at 200x and inlays at 600x magnification.

The Vpr positive staining cells in Figure 3A, 3B, 3D and 3F appear to be neurons based on cellular morphology. This is an interesting finding since there is a controversy over whether or not neurons can even be infected by HIV-1. This does however support the previous studies suggesting that Vpr can transduce into, and have a neurotoxic effect on neurons. The Vpr positive staining cells in Figure 3C and 3E are present near blood vessels, and based on morphology appear to be infiltrating macrophages. The presence of Vpr in infiltrating macrophages is anticipated since perivascular macrophages have been shown to be susceptible to HIV-1 infection (28). Perivascular macrophages have also been shown to be capable of supporting a productive HIV-1 infection within the brain (29).

Several negative controls were run to ensure the accuracy of the Vpr positive staining. 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 tissue. In performing the no primary antibody control, the primary antibody step is omitted, and all other steps of the technique remain the same. The no primary antibody controls (Figure 4A, 4C, and 4E) were negative, which supports the accuracy of the Vpr staining seen in Figure 3.

The second negative control used was a rabbit serum control. This control was run to show that the primary antibody was not reacting non-specifically with anything in the HIVE brain tissue. Since the primary antibody was rabbit polyclonal serum antibody specific for Vpr, normal rabbit serum (Vector Laboratories, Burlingame, CA) was used for this control. The tissue sections were incubated with normal rabbit serum in place of the primary antibody, at the same time, temperature, and dilution conditions. The rabbit serum controls (Figure 4B, 4D, and 4F) were negative, further supporting the validity of the Vpr positive staining in Figure 3.

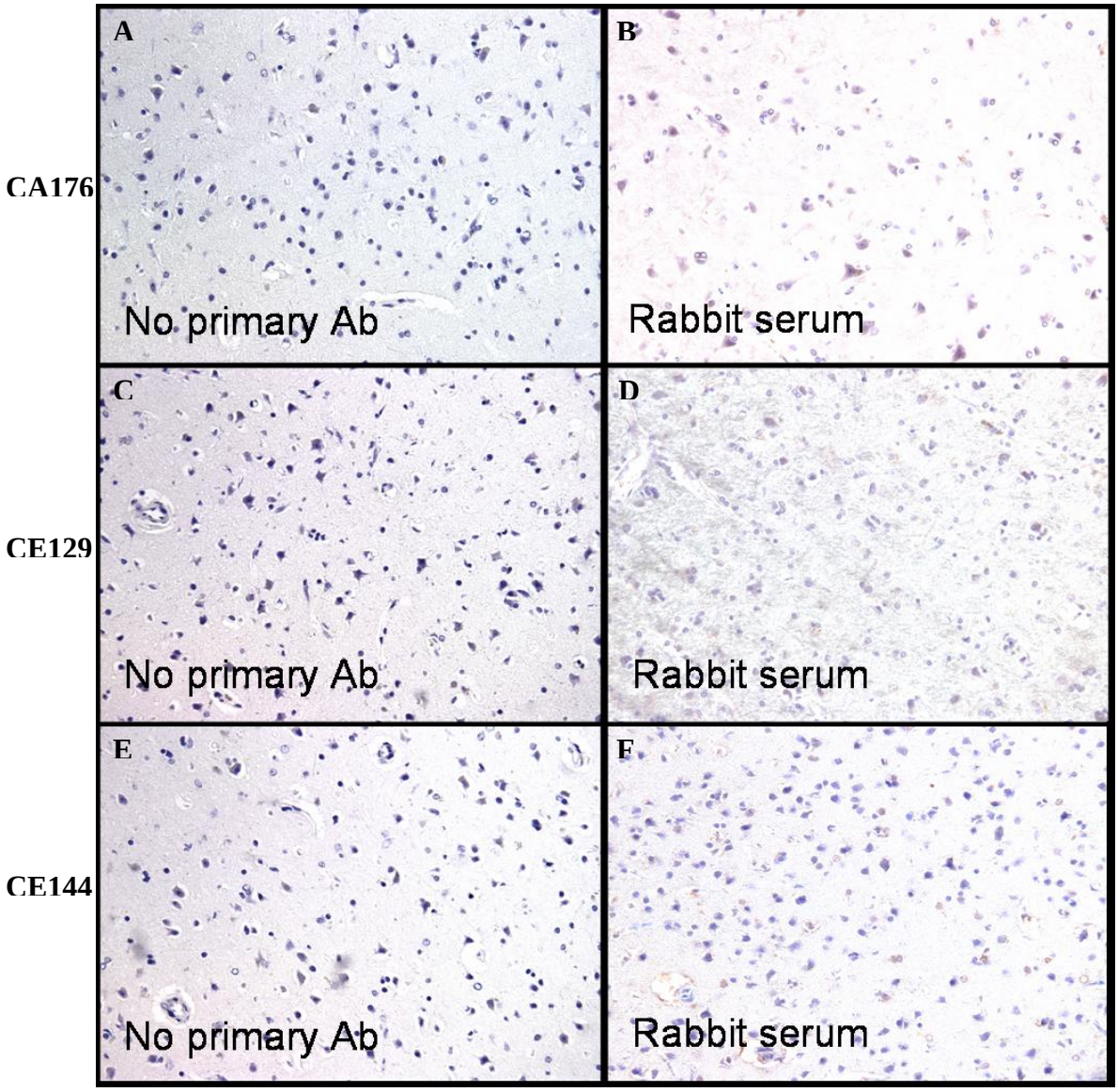

Figure 4: Immunohistochemistry negative controls

No primary antibody controls, in which the primary antibody step has been omitted, are negative (**A, C, and E**). Rabbit serum controls, in which normal rabbit serum was added to the tissues in place of the primary antibody, are also negative (**B, D, and F**). Pictures are a representative of all 8 HIVE patients. Pictures were taken at 200x magnification.

HIV seronegative patients were also used as negative controls. The 4 seronegative patients, each with a basal ganglia and a frontal cortex slide, were incubated with the Vpr specific antibody, and stained following the same protocol as was used on as the HIVE patients presented in Figure 3. The seronegative patients displayed no Vpr positive staining in either the basal ganglia (Figure 5 A and B) or in the frontal cortex (Figure 5 C and D). These results definitively support the specificity of the positive Vpr staining seen in the brain tissue of the HIVE patients.

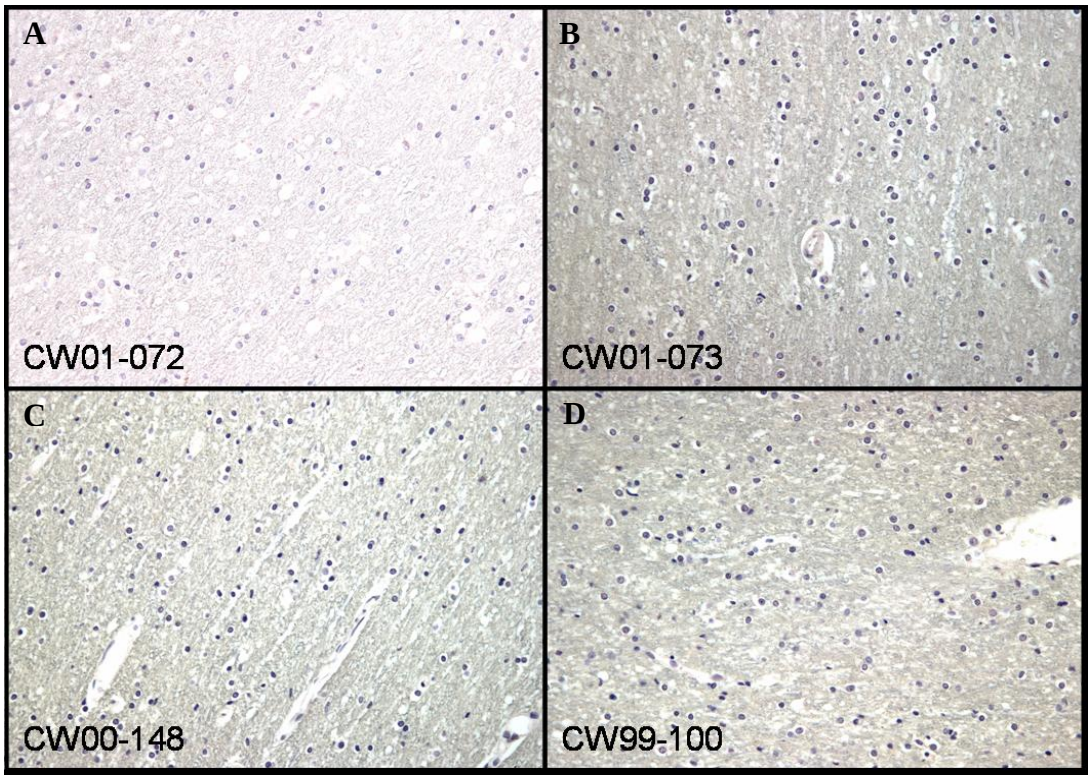

Figure 5: HIV Seronegative Vpr immunohistochemistry controls

HIV seronegative controls were run, at the same time as HIVE patients, for both the basal ganglia and frontal cortex. No Vpr positive staining was seen in any of the seronegative slides tested. (**A and B**) Representative of the 4 seronegative basal ganglia tissue sections tested. (**C and D**) Representative of the 4 seronegative frontal cortex sections tested. Pictures taken at 200x magnification.

4.1.4. Comparison of Vpr and p24 Staining in HIVE Brain Tissue

In order to compare one HIVE patient to another, it was necessary to create a way to quantify the positive Vpr and p24 immunohistochemical staining. To do this, each patient and tissue section was scanned for 10 random fields at 400x magnification, and all the positive staining cells were counted. The sums for each field were then added together, and compared to a ranking scale (0-25 total positive cells = 1+, 26-50 total positive cells = 2+, 51-75 total positive cells = 3+, 76-100 positive cells = 4+). The summary of these quantifying results can be seen in Table 2. When comparing the CSF and plasma viral loads (Table 1) to the Vpr quantification results, no apparent linear association was seen. However, the two patients with the highest plasma and CSF viral loads (CE129 and 10017) did exhibit the highest amounts of Vpr staining.

Table 2: Summary of Vpr and p24 immunohistochemistry staining

10 random fields for each tissue section were scanned at 400x, 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. (0-25 total positive cells = 1+, 26-50 total positive cells = 2+, 51-75 total positive cells = 3+, 76-100 total positive cells = 4+)

Patient ID	HIV status	Paraffin Section	rVpr +	mp24 +
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	—	—
CW01-073-B	Seronegative	frontal cortex	—	—
CW00-148-B	Seronegative	frontal cortex	—	—
CW99-100-B	Seronegative	frontal cortex	—	—
CW01-072-G	Seronegative	basal ganglia	—	—
CW01-073-G	Seronegative	basal ganglia	—	—
CW00-148-G	Seronegative	basal ganglia	—	—
CW99-100-B	Seronegative	basal ganglia	—	—

Taken together, these results indicate that HIV-1 Vpr is detectable in the basal ganglia and frontal cortex of HIVE patients. Additionally, staining for p24 antigen verified the presence of HIV-1 virus in the HIVE brain tissue. The cell morphology of those cells positive for Vpr indicate that Vpr may potentially be present in macrophages and neurons of the HIVE brain tissue. These results will be confirmed using cell type specific markers as outlined in Specific Aim #2.

4.2. DETECTION OF VPR IN SPECIFIC CELL TYPES OF HIVE BRAIN TISSUE

The results of Specific Aim #1 indicated the presence of Vpr in detectable levels in the brain tissue of HIVE patients. Additional staining for p24 antigen further confirmed the presence of HIV-1 virus in both the basal ganglia and frontal cortex of these patients. During the course of HIV infection in the perivascular region of the brain, macrophages, and astrocytes come into direct contact with cells that are infected with HIV and have entered the CNS through the blood. Macrophages are not only susceptible to infection by HIV, but are also capable of supporting a productive HIV infection (18). Astrocytes have also been shown to be susceptible to infection by HIV (30). Significant neuronal loss and damage is observed in HIVE patients (55). For these reasons I have chosen to examine macrophages, astrocytes and neurons for the presence of Vpr positive staining. Initial results in Specific Aim #1 indicated two morphologically distinct cell types positive for Vpr. Using specific markers for macrophages, astrocytes, and neurons, I will further confirm the cell types that are positive for Vpr.

4.2.1. Vpr Detection in Macrophages of HIVE Brain Tissue

Circulating monocytes enter the brain to replace those macrophages that have become subject to insult by vascular problems or protein build up due to neurodegeneration (18). These monocytes then mature into the infiltrating and perivascular macrophages found in the brain (16). While the replenishment of perivascular macrophages by circulating monocytes is necessary for the immunological protection of the brain, it also presents a potential pathway for pathogens to enter the brain. Intracellular pathogens may use circulating monocytes as a vehicle

by which to pass the blood-brain barrier. HIV is believed to enter the brain in just this manner, by what is known as the "Trojan horse" hypothesis (27). The "Trojan horse" hypothesis suggests that HIV enters the brain inside infected monocytes, which later differentiate into brain macrophages. Infiltrating and perivascular macrophages are the most commonly infected cell types of the CNS during any stage of HIV infection (28). Infiltrating and perivascular macrophages have also been shown to be capable of sustaining a productive HIV infection (29). It has been suggested that brain macrophages also serve as a cellular reservoir for HIV-1 (110). A substantial accumulation of brain macrophages has also been shown to occur concurrent with the inflammation present in HIVE (97). Free Vpr released from infected macrophages has been shown to be capable of transduction into, followed by killing of, bystander cells not normally infected by HIV (72). In addition, Vpr plays an important role in sustaining HIV infection in differentiated macrophages. Therefore, based on previous studies linking Vpr to the neuropathogenic role of infected brain macrophages, Vpr should be detectable in the macrophages of the HIVE encephalitic brain tissue.

To address whether Vpr was present in the infiltrating and perivascular macrophages of the HIVE brain tissue, double label immunohistochemistry was performed. Following tissue and slide preparations, as described in the materials and methods, the tissue sections were incubated with a rabbit polyclonal Vpr specific antibody (1:100), and a macrophage marker, mouse monoclonal CD68 (1:25) for 12 hours at 4ºC. Next, tissue sections were incubated with a mixture of two secondary antibodies, a goat anti-rabbit Cy2 (1:200), and a goat anti-mouse Cy3 (1:600), for 2 hours at 37ºC. The tissue sections were then viewed by immunofluorescent laser confocal microscopy, and examined for the presence of double positive cells for both Vpr and CD68.

The results presented in Figures 6, 7, and 8, indicate that Vpr positive (green) staining was seen in cells which also stained positive for CD68 (red). The three patients, CA176 (Figure 6), CE129 (Figure 7), and CE144 (Figure 8), were included in this study based on the availability of both the basal ganglia and frontal cortex of these patients.

While both perivascular and infiltrating macrophages can be seen adjacent to the brain microvasculature, they can be distinguished form one another based on morphology. Perivascular macrophages appear flat and elongated when compared to the more rounded appearance of an infiltrating macrophage. In the top row of Figure 6, patient CA176 is displaying Vpr positive perivascular macrophages (arrow), based on CD68 staining, surrounding a blood vessel in the basal ganglia of the HIVE brain tissue. Vpr positive infiltrating macrophages can be seen around a blood vessel in the frontal cortex of patient CA176, shown in the second row of Figure 6 (arrowhead). Negative controls, in which the primary antibody step was omitted, but all other staining steps were kept the same, are also shown in the bottom row of Figure 6. Controls in both the frontal cortex and basal ganglia show no red or green, indicating that the staining described for Vpr and CD68 is not due to nonspecific staining of the conjugated secondary antibodies. These results show the presence of Vpr in infiltrating and perivascular macrophages in patient CA176.

Similar results were seen with patient CE129, as shown in Figure 7. The cells shown in Figure 7 in both the basal ganglia and frontal cortex are staining positive for both Vpr and CD68 (colocalization shown as yellow). The cells in the basal ganglia and frontal cortex of patient CE129 are surrounding blood vessels, and appear to be infiltrating macrophages based on morphology (arrowheads). These results show the presence of Vpr positive infiltrating macrophages in patient CE129.

Patient CE144 also displayed Vpr positive staining in cells positive for CD68 (Figure 8). The top row of pictures in Figure 8, from the basal ganglia of patient CE144, reveals Vpr positive infiltrating macrophages, based on morphology, adjacent to a blood vessel. In the second row of pictures in Figure 8, taken from the frontal cortex of patient CE144, Vpr positive perivascular macrophages can be seen surrounding a blood vessel. These results display the presence of Vpr positive infiltrating and perivascular macrophages in patient CE144.

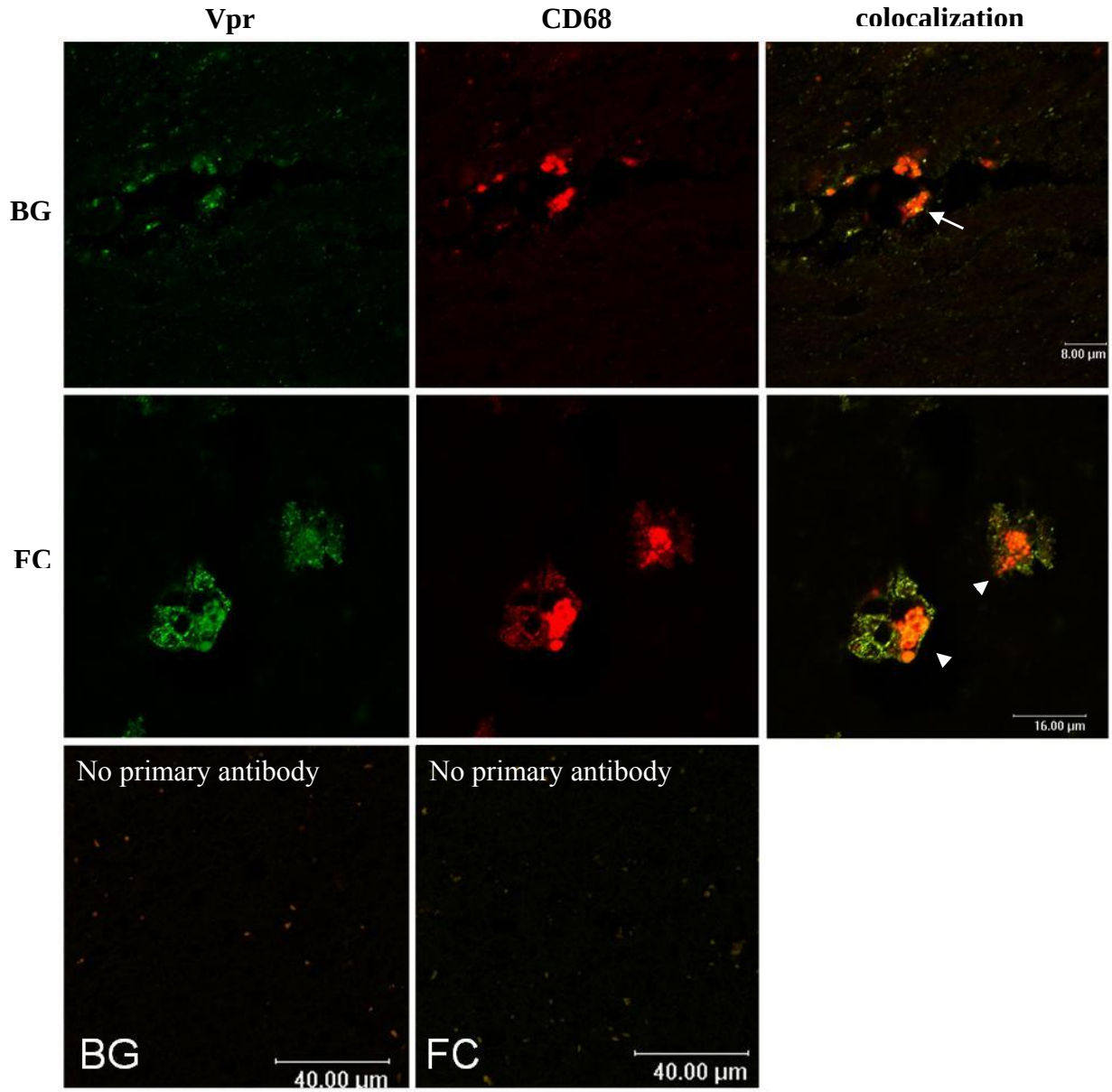

Figure 6: Vpr and CD68 double label immunohistochemistry for patient CA176

Primary antibodies against Vpr (green) and CD68 (red) were used to show the presence of Vpr in the infiltrating and perivascular macrophages of this patient. Colocalization of Vpr and CD68 (yellow) can be seen in the far right column. No primary antibody negative controls are also shown for both the (BG) basal ganglia (FC) frontal cortex. Pictures were taken at 600x magnification on an immunofluorescent laser confocal microscope. Perivascular macrophages are indicated with arrows, and infiltrating macrophages are indicated with arrowhead.

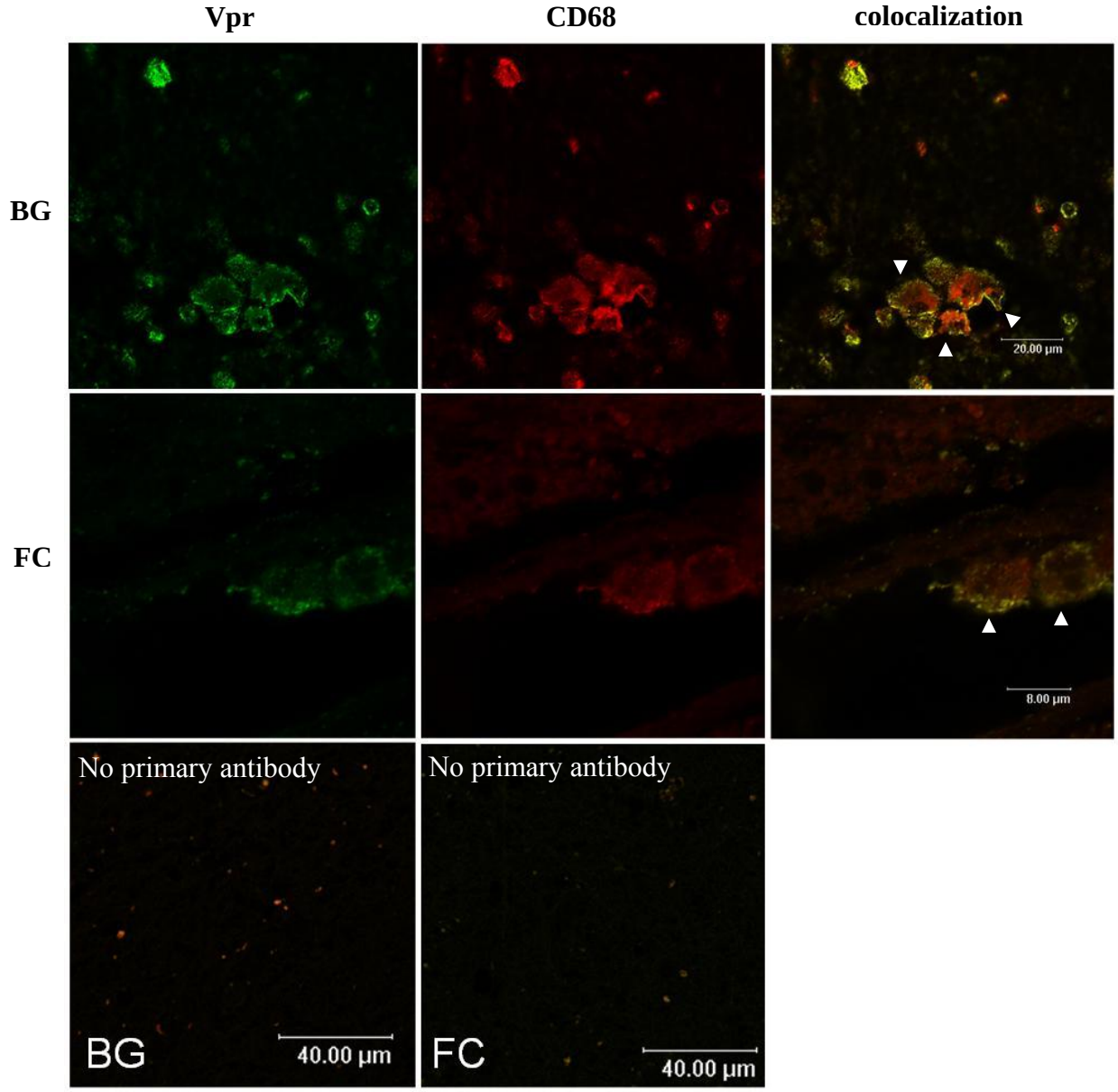

Figure 7: Vpr and CD68 double label immunohistochemistry for patient CE129

Primary antibodies against Vpr (green) and CD68 (red) were used to show the presence of Vpr in the infiltrating macrophages (arrowheads) of this patient. Colocalization of Vpr and CD68 (yellow) can be seen in the far right column. No primary antibody negative controls are also shown for both the (BG) basal ganglia (FC) frontal cortex. Pictures were taken at 600x magnification on an immunofluorescent laser confocal microscope.

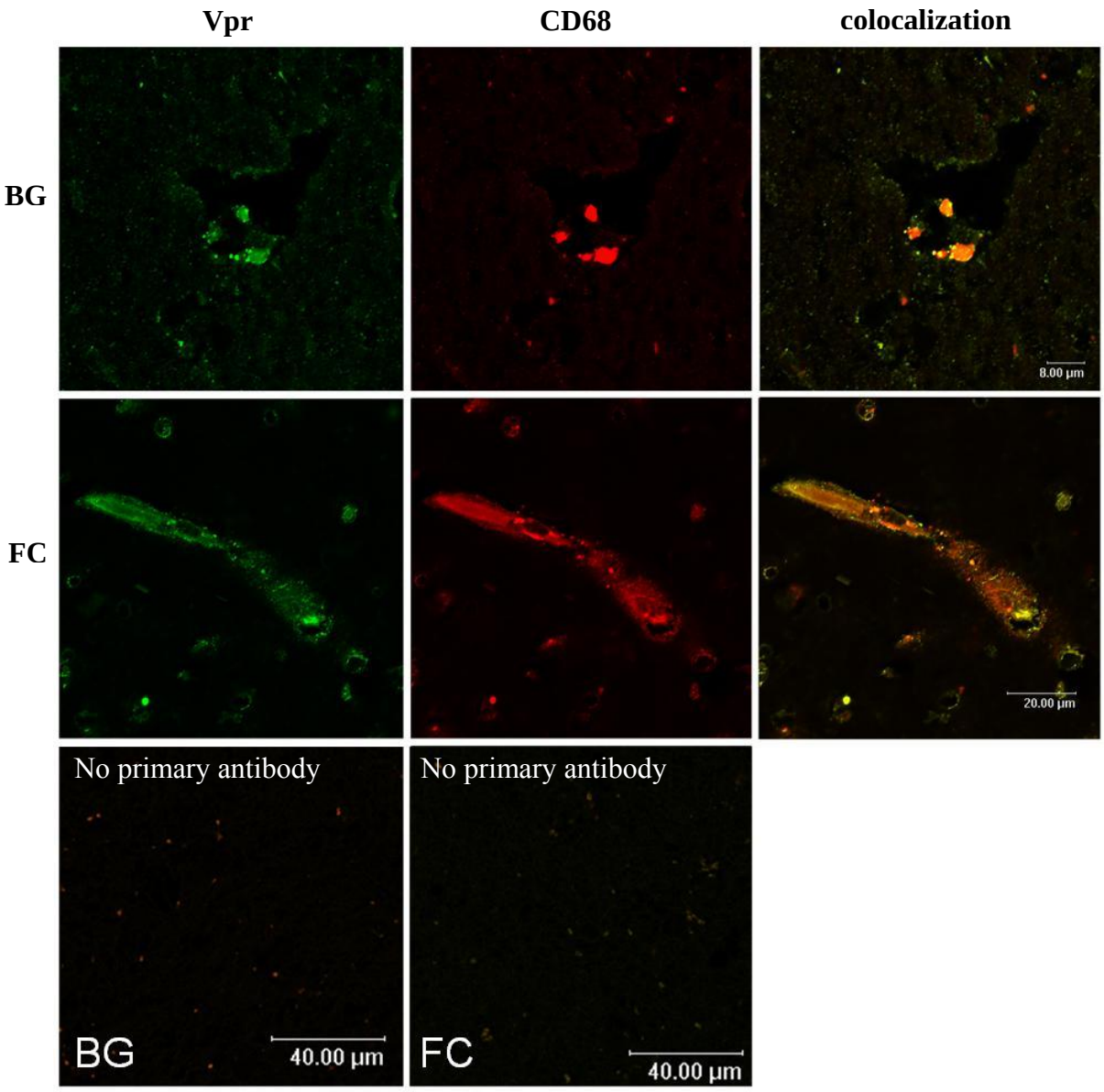

Figure 8: Vpr and CD68 double label immunohistochemistry for patient CE144

Primary antibodies against Vpr (green) and CD68 (red) were used to show the presence of Vpr in the infiltrating (arrowheads) and perivascular (arrows) macrophages of this patient. Colocalization of Vpr and CD68 (yellow) can be seen in the far right column. No primary antibody negative controls are also shown for both the (BG) basal ganglia (FC) frontal cortex. Pictures were taken at 600x magnification on an immunofluorescent laser confocal microscope.

HIV seronegative controls were also run as negative controls in parallel with the HIVE patients. Four HIV seronegative patients, each with both basal ganglia and frontal cortex tissue sections available were tested. The tissue sections were incubated with the Vpr specific and CD68 specific antibodies as previously described, and results were visualized by immunofluorescent laser confocal microscopy. No Vpr positive staining was seen in any of the tissue sections for the seronegative patients (Figure 9 Top Row). Previous studies have indicated that increased numbers of infiltrating macrophages can be seen during HIVE (97). Similar results were seen in this study when comparing the amount of infiltrating macrophages in the HIVE patients to the seronegative patients (Figure 9). The HIVE patients (Figure 9 Bottom Row) displayed increased numbers of infiltrating macrophages when compared to the seronegative controls (Figure 9 Top Row). These results were consistent throughout all of the eight HIVE patients. In addition, the increased amount of infiltrating macrophages could be seen in both the basal ganglia and frontal cortex of the HIVE patients.

Collectively, these results indicate that Vpr is present in detectable amounts in the infiltrating and perivascular macrophages of HIVE patients. The positive staining macrophages can be found in both the basal ganglia and frontal cortex of the HIVE brain tissue. In addition, the HIVE brain tissue displayed an increased amount of infiltrating macrophages when compared to HIV seronegative patients.

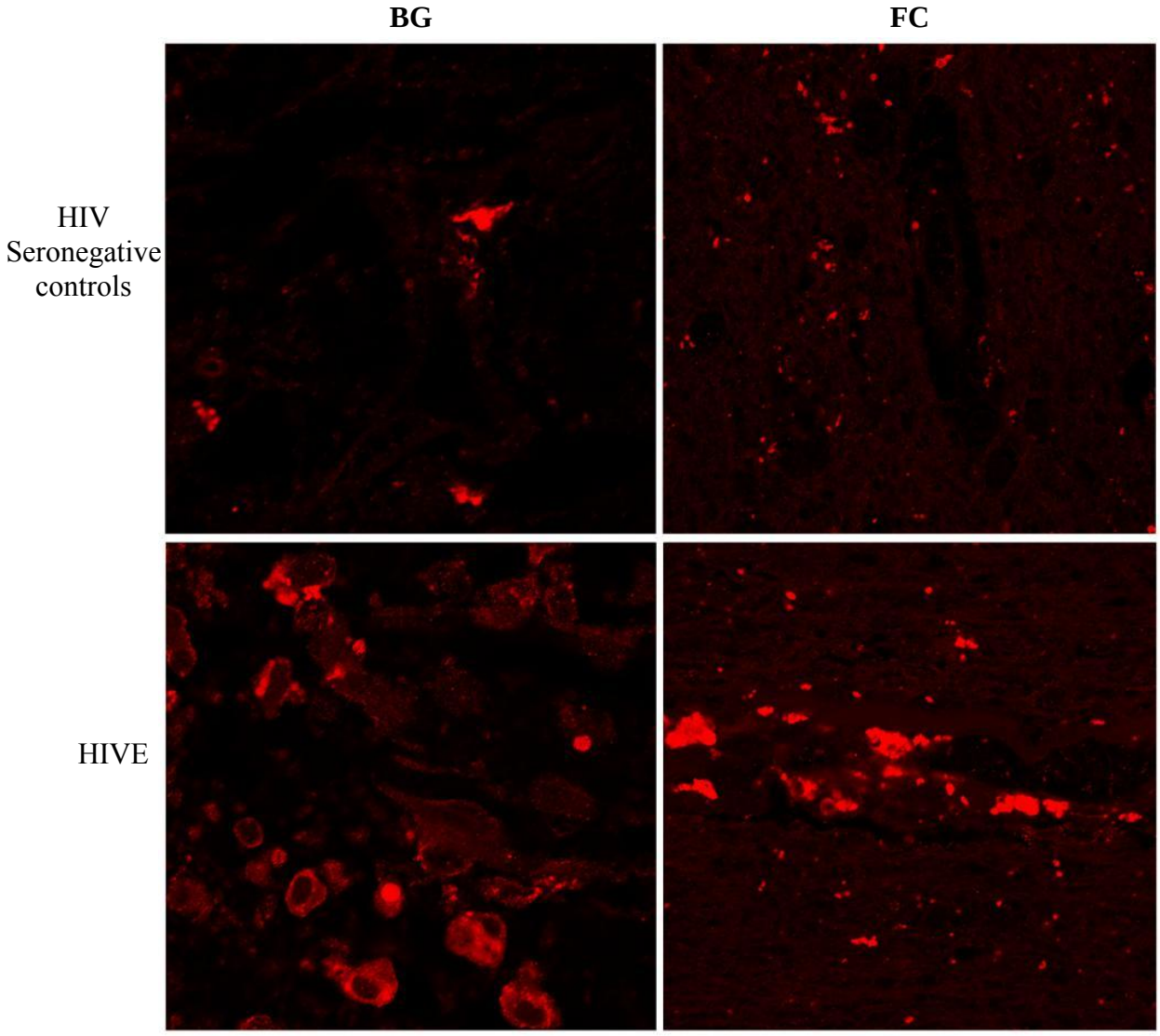

Figure 9: HIV seronegative controls for Vpr and CD68 double label immunohistochemistry

(**Top Row**) HIV seronegative controls were incubated with a mixture of a rabbit polyclonal Vpr specific antibody (green) and a mouse monoclonal CD68 macrophage specific antibody (red). Seronegative pictures revealed no Vpr positive staining (representative of 4 seronegative patients for both the basal ganglia and frontal cortex). (**Bottom Row**) HIVE patients displayed increased numbers of infiltrating macrophages when compared to HIV seronegative control patients. HIVE patients are shown here for just CD68 (red) staining. Pictures were taken at 600x magnification.

4.2.2. Absence of 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 (111). Through the release of soluble factors and contact with brain microvascular endothelial cells, astrocytes regulate the permeability of the blood-brain barrier (18). Astrocytes are also responsible for providing neurons the necessary metabolites to regulate ion and neurotransmitter concentrations (111). Astrocytes have been shown to be susceptible to HIV infection through a CD4-independent, human mannose receptor (hMR)-dependent mechanism (30). However, HIV-1 infection in astrocytes can only be detected through sensitive techniques that involve HIV-1 RNA or proviral DNA detection (92). It has been suggested that astrocytes are not only a cellular reservoir for HIV-1 infection in the brain, but also the mediators of HIV-1 induced neuronal damage (111). Furthermore, only restricted or infrequent HIV-1 productive infection has been shown in astrocytes; thus HIV-1 proteins, including accessory proteins, are only present in astrocytes in minimal amounts. Vpr induced astrocytic cell death has been observed by both necrotic and apoptotic mechanisms in rat astrocytic cell lines *in vitro* (91). Based on the suggested role of Vpr in astrocytic cell death , it is important to determine whether Vpr is present in detectable amounts in astrocytes of the HIVE brain tissue.

To address whether Vpr was present in detectable amounts in the astrocytes of the HIVE brain tissue, double label immunohistochemistry was performed. Tissue sections from the basal ganglia and frontal cortex of HIVE patients were prepared as described in the materials and methods, followed by an incubation with a rabbit polyclonal Vpr specific antibody (1:100), and an astrocytic marker, mouse monoclonal Glial Fibrillary Acidic Protein (GFAP) (1:100) for 12 hours at 4ºC. The tissue sections were then incubated with a mixture of two secondary

antibodies, a goat anti-rabbit Cy2 (1:200), and a goat anti-mouse Cy3 (1:600) for 2 hours at 37ºC. Double labeled sections were viewed by immunofluorescent laser confocal microscopy, and examined for the presence of double positive cells for both Vpr and GFAP. Results of the double label staining for Vpr and GFAP are presented in Figures 10, 11, and 12.

Patient CA176 (Figure 10) did not show positive Vpr staining in those cells positive for GFAP. In the basal ganglia of CA176 (Figure 10 Top Row) GFAP (red) staining can be seen surrounding a blood vessel. Similar brilliant staining can be seen for GFAP in the frontal cortex of CA176 (Figure 10 Second Row), indicating the presence of astrocytes in these sections. These results indicate that Vpr was not present in detectable amounts in the astrocytes of HIVE patient CA176.

Similar results were seen for patient CE129 (Figure 11). Pronounced GFAP staining can be seen in the basal ganglia (Figure 11 Top Row) of patient CE129, but the Vpr staining again does not exhibit colocalization with GFAP. In the frontal cortex of CE129 (Figure 11 Second Row), pronounced GFAP staining can be seen surrounding a blood vessel. The positive Vpr staining seen in the frontal cortex fails to colocalize with GFAP as well. Therefore, Vpr is not present in the astrocytes of either the basal ganglia or the frontal cortex of HIVE patient CE129.

The results for HIVE patient CE144 can be seen in Figure 12. Radiant GFAP positive staining can be seen surrounding a blood vessel in the basal ganglia of patient CE144 (Figure 12 Top Row). Positive Vpr staining can also be seen around the blood vessel (Figure 12 Top Row), but not in the same cells staining positive for GFAP. Likewise, GFAP staining can be seen surrounding a blood vessel in the frontal cortex of patient CE144 (Figure 12 Second Row). While positive Vpr staining is present around this blood vessel as well, it is not present in those cells positive for GFAP.

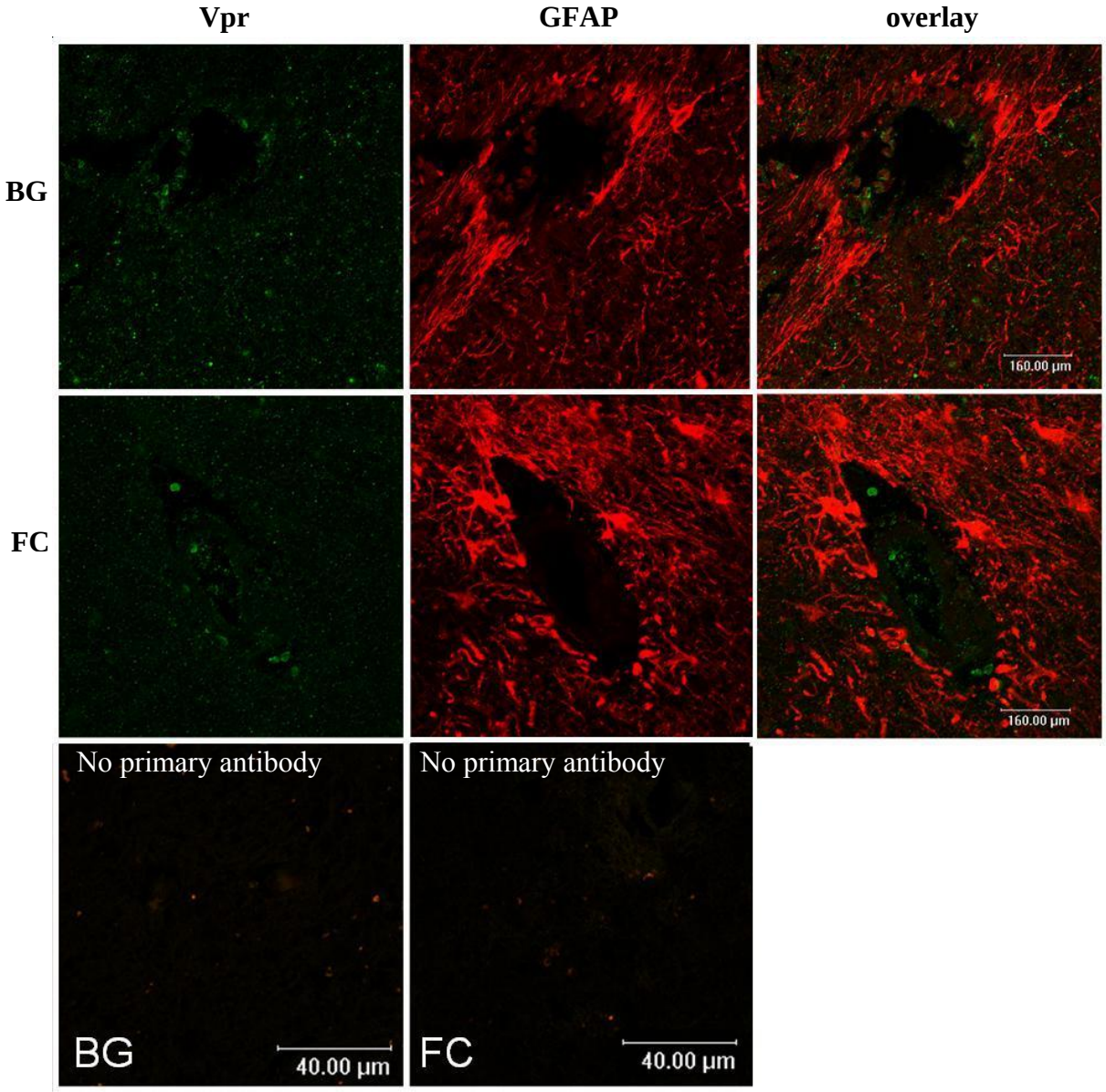

Figure 10: Vpr and GFAP double label immunohistochemistry for patient CA176

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 colocalization was seen in either the basal ganglia (**BG**) or frontal cortex (**FC**) of this patient. No primary antibody negative controls are also shown (**Bottom Row**). Pictures were taken at 600x magnification.

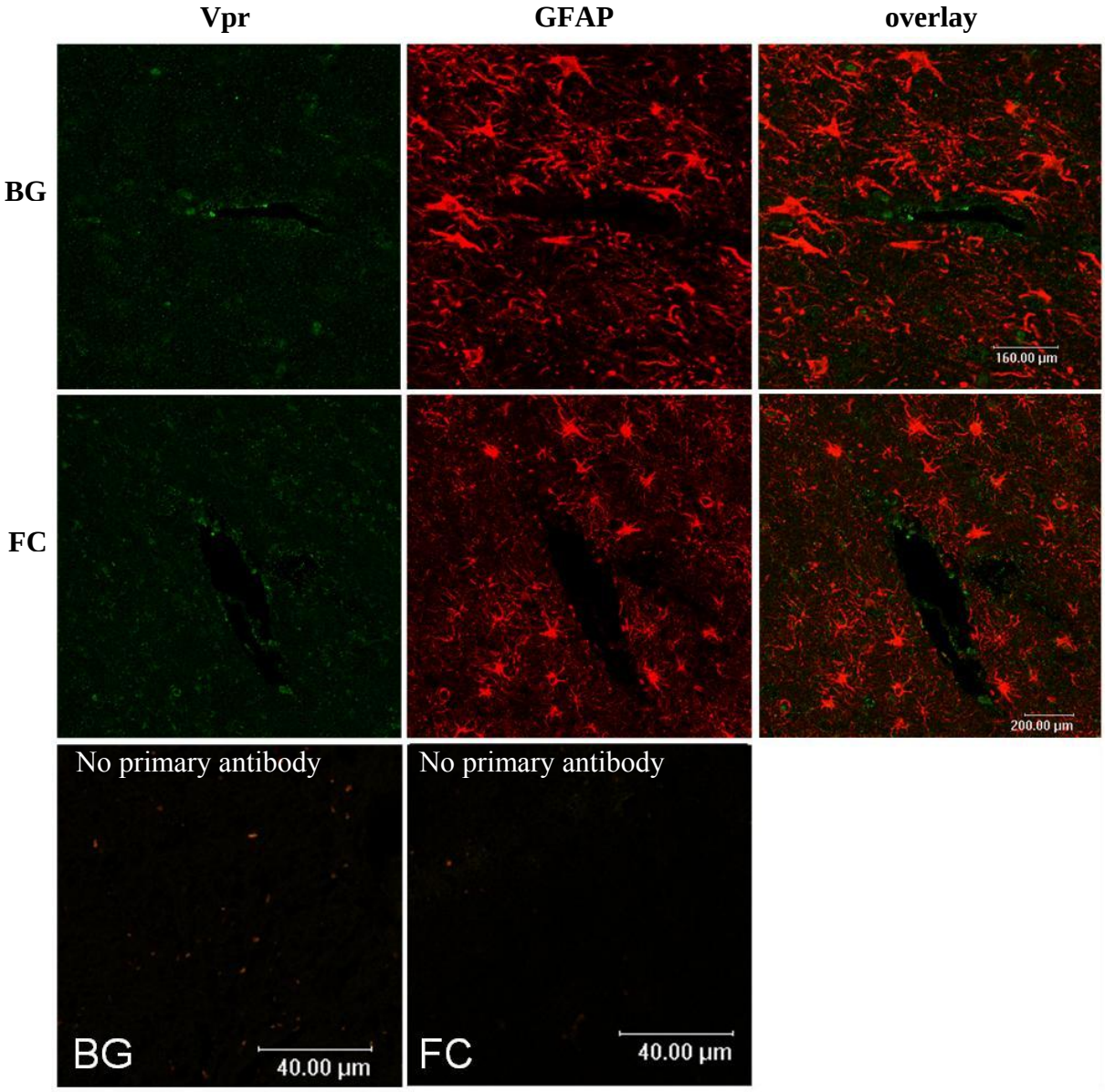

Figure 11: Vpr and GFAP double label immunohistochemistry for patient CE129

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 colocalization was seen in either the basal ganglia (**BG**) or frontal cortex (**FC**) of this patient. No primary antibody negative controls are also shown (**Bottom Row**). Pictures were taken at 600x magnification.

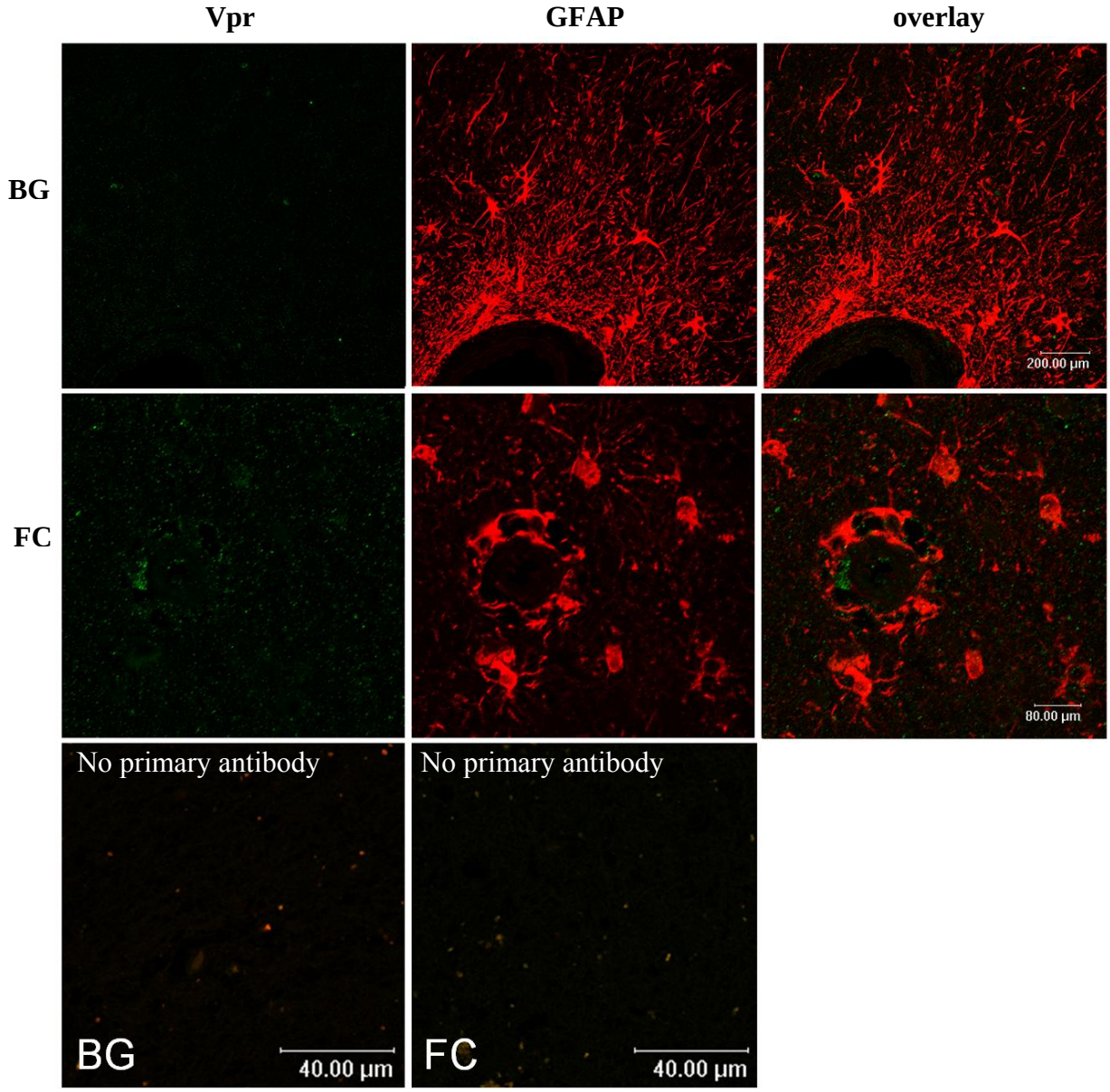

Figure 12: Vpr and GFAP double label immunohistochemistry for patient CE144

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 colocalization was seen in either the basal ganglia (**BG**) or frontal cortex (**FC**) of this patient. No primary antibody negative controls are also shown (**Bottom Row**). Pictures were taken at 600x magnification.

HIV seronegative patients were used as negative controls, and run along side the HIVE patients in the double label immunohistochemistry for Vpr and GFAP. The basal ganglia and frontal cortex of four HIV seronegative patients were tested. The tissue sections were incubated with the Vpr specific and GFAP astrocyte specific antibodies as described previously in this section. The results were visualized by immunofluorescent laser confocal microscopy, and can be seen in Figure 13. No Vpr staining was seen in any of the brain tissue sections of the four seronegative controls (Figure 13 Top Row). During HIVE the metabolic activity of astrocytes increases, and the astrocytes themselves proliferate intensely in a process known as astrogliosis (112). This phenomenon can be seen in the bottom row of Figure 13. The HIVE patients display pronounced astrogliosis when compared to HIV seronegative controls.

Collectively, these results indicate that Vpr is not present in detectable amounts in astrocytes of the HIVE brain tissue. These results were found in both the basal ganglia and frontal cortex on the HIVE patients. Additionally, the HIVE patients display pronounced astrogliosis in the basal ganglia and frontal cortex when compared to HIV seronegative controls.

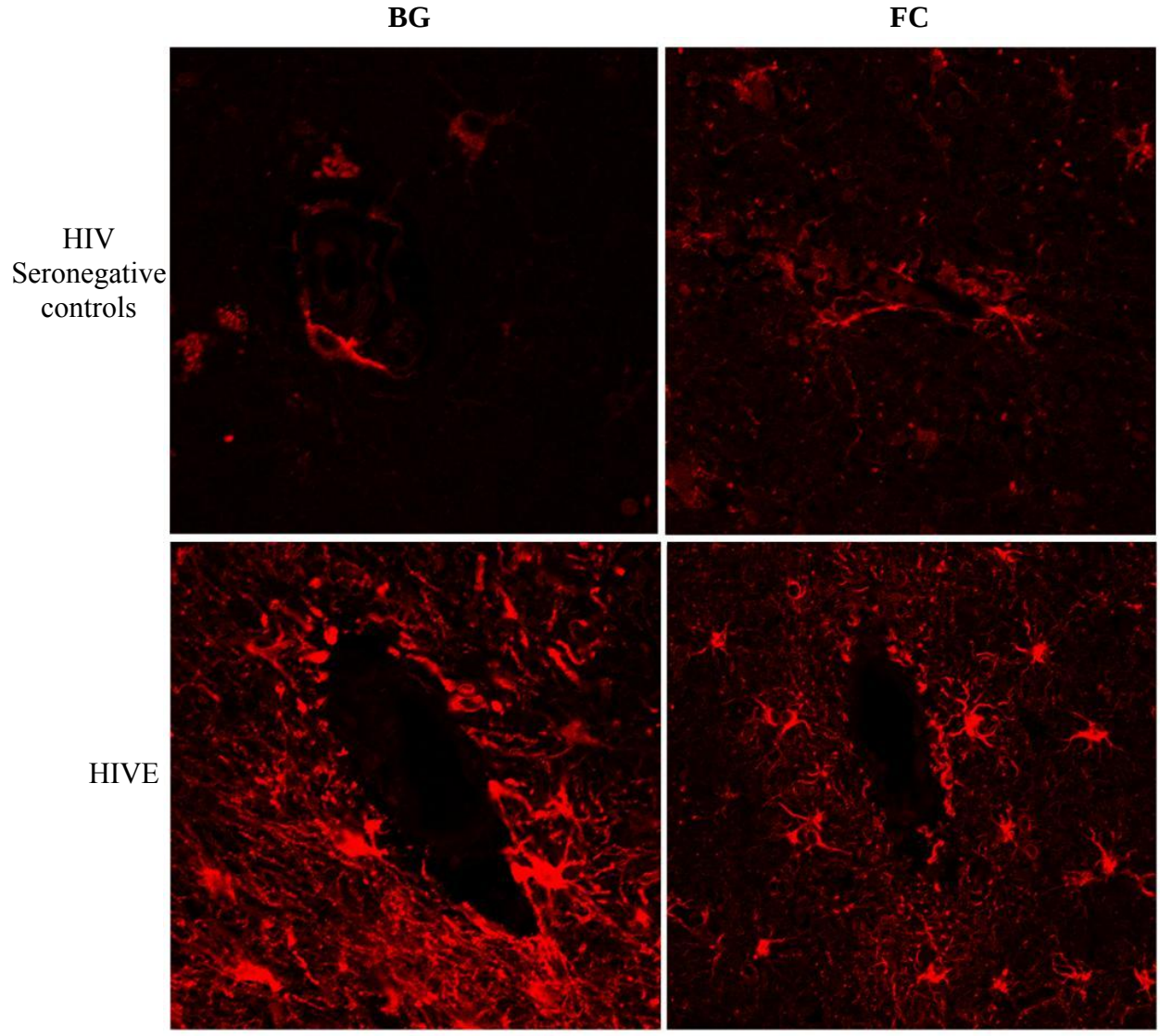

Figure 13: HIV seronegative controls for Vpr and GFAP double label immunohistochemistry

(**Top Row**) HIV seronegative controls were incubated with a mixture of a rabbit polyclonal Vpr specific antibody (green) and a mouse monoclonal GFAP astrocytes specific antibody (red). Seronegative patients displayed no Vpr positive staining (representative of 4 seronegative patients for both the basal ganglia and frontal cortex). (**Bottom Row**) HIVE patients displayed pronounced astrogliosis when compared to HIV seronegative control patients. HIVE patients are shown here for just GFAP (red) staining. Pictures were taken at 600x magnification.

4.2.3. Vpr Detection in Neurons of HIVE Brain Tissue

Neurons are the main effectors of cognitive and motor function in the brain (18). Both neuronal loss and damage are seen during the progression of HIVE (113). The neurodegeneration seen in HIVE is predominantly attributable to apoptosis (34). It has been shown by several groups that HIV-1 viral proteins can cause injury or death of neurons (31-33). Free Vpr has been shown to be able to transduce through intact cytoplasmic membranes into cells that are not normally infected by HIV-1 and cause death of these bystander cells (72). Additionally, Vpr has specifically been shown to be capable of transduction into non-proliferating cells, including neurons (114). Once inside the cell, Vpr has been shown to induce apoptosis of human neuronal precursor cells, as well as mature differentiated neurons, through a caspase 8 dependent mechanism (54, 83). Furthermore, Vpr has been shown to form cation-selective ion channels in planar lipid bilayers through interactions with its amino-terminal region, disrupting the ionic gradient and causing a large inward current and cell death in neurons (89, 90). Therefore, based on the previous findings of the neuropathogenic effects of Vpr, Vpr should be present in detectable amounts in neurons of the HIVE brain tissue.

To determine whether Vpr was present in detectable amounts in the neurons of the HIVE brain tissue, double label immunohistochemistry was performed. Following tissue preparations as described in the materials and methods section, HIVE patient tissue sections were incubated with a rabbit polyclonal Vpr specific antibody (1:100), and a mouse monoclonal SMI312 neuron specific marker (1:200), for 12 hours at 4ºC. SMI312 is a cocktail of monoclonal IgG_1 antibodies to phosphorylated neurofilaments, which selectively marks axons and neuronal cell bodies in tissue sections. The tissue sections were then incubated with a mixture of two

secondary antibodies, a goat anti-rabbit Cy2 (1:200), and a goat anti-mouse Cy3 (1:600), for 2 hours at 37ºC. Staining was then visualized by immunofluorescent laser confocal microscopy, and examined for the presence of double positive cells for both Vpr and GFAP, the results of which are presented in Figures 14, 15, and 16.

Patient CA176 (Figure 14) displayed Vpr positive staining in neurons present in both the basal ganglia and frontal cortex. In the basal ganglia of CA176 (Top Row of Figure 14), the Vpr positive staining can be seen around the edges of the SMI312 positive cell (indicated by arrow). In contrast, the SMI312 positive cells seen in the frontal cortex (Second Row Figure 14) displays positive Vpr staining throughout the neuronal cell body. These results indicate that Vpr is present in a small number of SMI312 positive cells found in patient CA176.

Similar Vpr positive staining was also observed in SMI312 positive cells in patient CE129 (Figure 15). As shown in the top row of Figure 15, Vpr positive staining was seen around the edge of the cells staining positive for SMI312. However, the Vpr positive staining seen in the frontal cortex (Figure 15 Second Row) was present in the neuronal cell body. The results from patient CE129 displayed a small percent of SMI312 positive cells that also stained positive for Vpr.

In Figure 16, the Vpr and SMI312 double positive cells can be seen in both the basal ganglia and frontal cortex of patient CE144. In the basal ganglia (Figure 16 Top Row), positive Vpr staining can be seen throughout the cell body of the cell staining positive for SMI312. The double positive cell seen in the frontal cortex of patient CE144 (Figure 16 Second Row) displays bright Vpr positive staining throughout the neuronal cell body. These results indicate that there is a small population of Vpr and SMI312 double positive cells present in patient CE144.

HIV seronegative patients served as negative controls, and were run in parallel with the HIVE patients for the double label immunohistochemistry with Vpr and SMI312. The basal ganglia and frontal cortex of four HIV seronegative patients were tested. The tissue sections were incubated with the Vpr specific and SMI312 neuron specific antibodies as described previously in this section. The results were visualized by immunofluorescent laser confocal microscopy, and can be seen in Figure 17. No Vpr positive staining was seen in any of the brain tissue sections of the four seronegative controls. As an additional control, no primary antibody controls were also used as negative controls for the double label immunohistochemistry with Vpr and SMI312 (Bottom Row of Figures 14, 15 and 16). For these controls, the double label staining protocol was carried out for HIVE patient samples with the omission of the primary antibody incubation step.

Collectively, these results indicate the presence of Vpr in neurons of the HIVE brain tissue. In all three patients, 2-5% of the cells staining positive for SMI312 were also staining positive for Vpr. The Vpr positive neurons were seen in both the basal ganglia and frontal cortex of the HIVE patients. These results were surprising due to the fact that the general belief of the HIVE research community is that neurons are not susceptible to infection by HIV. The results of this study would then indicate that free Vpr entered the neurons by transduction.

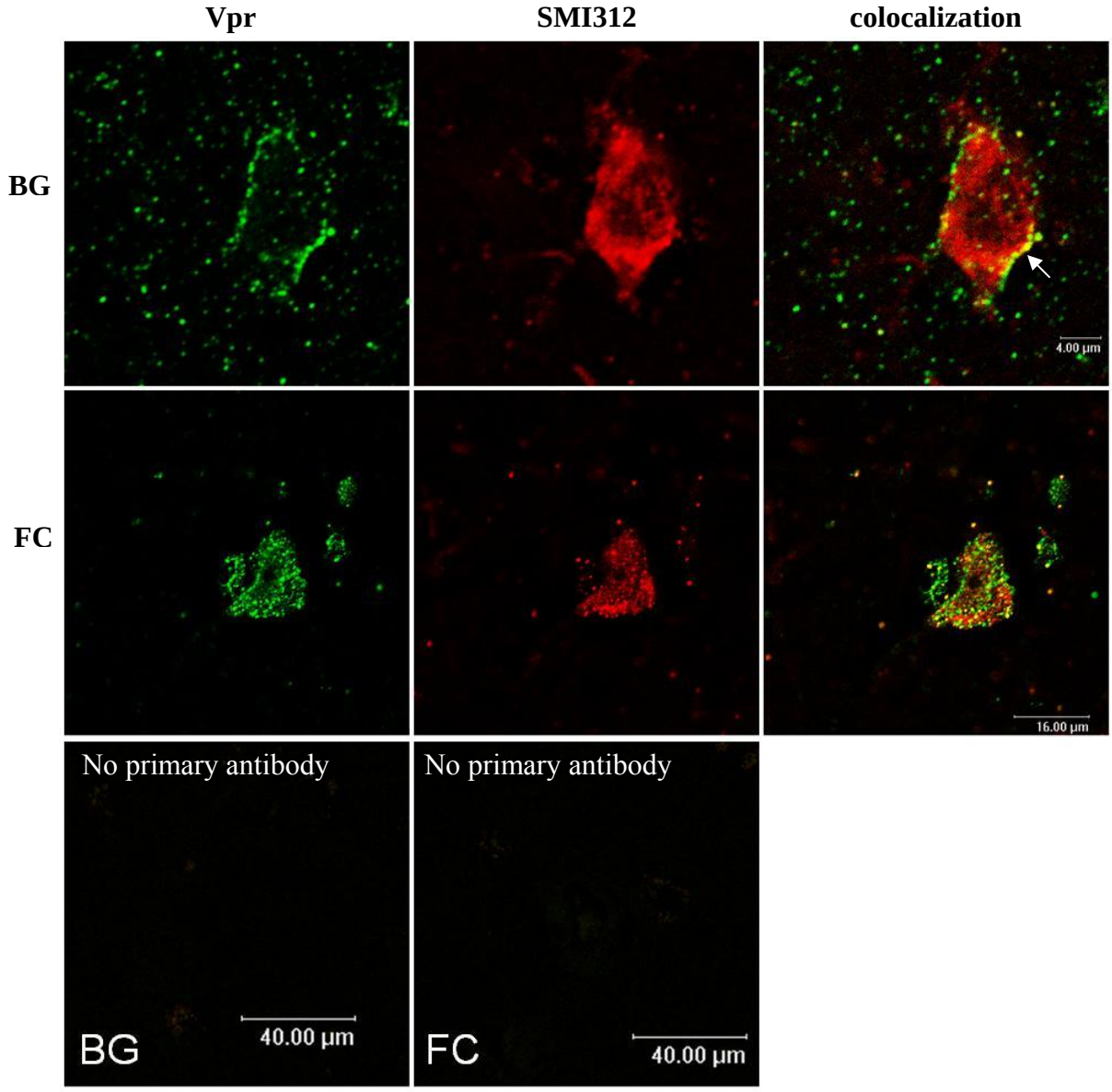

Figure 14: Vpr and SMI312 double label immunohistochemistry for patient CA176

Primary antibodies to Vpr (green) and SMI312 (red) were used to determine if Vpr was present in the neurons of the HIVE brain tissue. Vpr colocalization (yellow) of Vpr and SMI312 can be seen in the far right column for both the basal ganglia (**BG**) and frontal cortex (**FC**) of this patient. The arrow seen in the top row indicates the positive Vpr staining seen on the edge of the SMI312 positive cell. No primary antibody negative controls are also shown (**Bottom Row**). Pictures were taken at 600x magnification

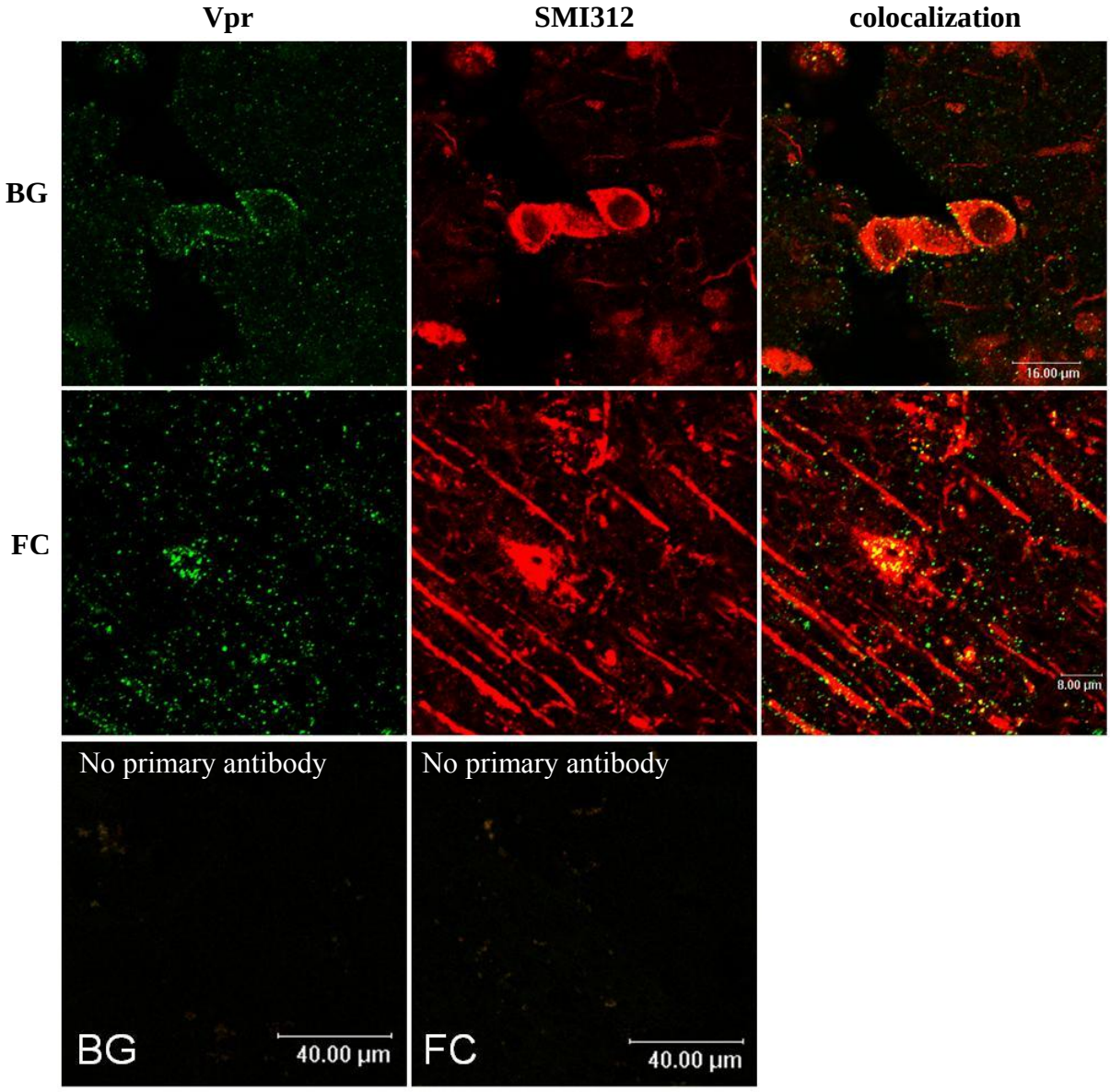

Figure 15: Vpr and SMI312 double label immunohistochemistry for patient CE129

Primary antibodies to Vpr (green) and SMI312 (red) were used to determine if Vpr was present in the neurons of the HIVE brain tissue. Vpr colocalization (yellow) of Vpr and SMI312 can be seen in the far right column for both the basal ganglia (**BG**) and frontal cortex (**FC**) of this patient. No primary antibody negative controls are also shown (**Bottom Row**). Pictures were taken at 600x magnification

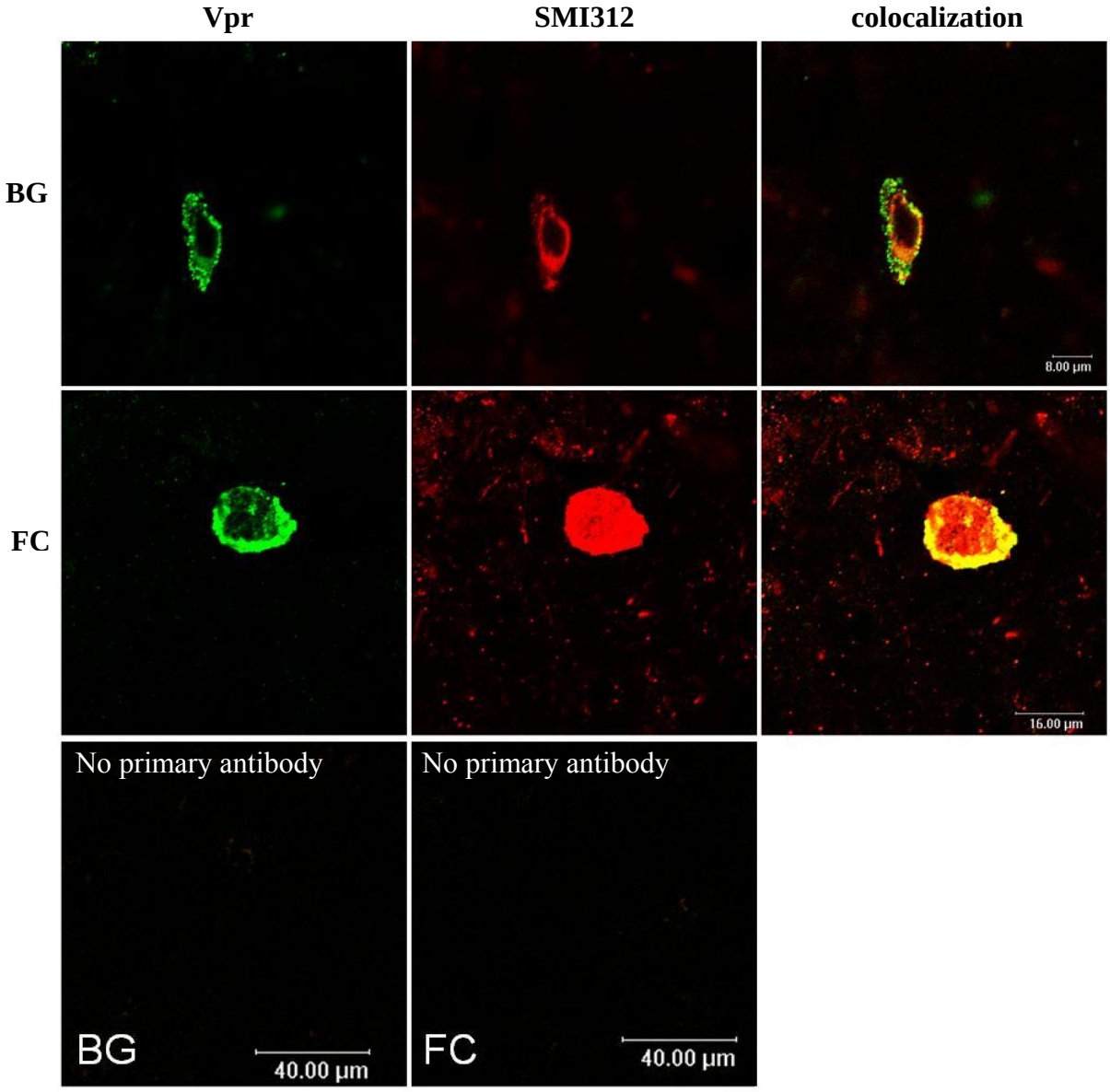

Figure 16: Vpr and SMI312 double label immunohistochemistry for patient CE144

Primary antibodies to Vpr (green) and SMI312 (red) were used to determine if Vpr was present in the neurons of the HIVE brain tissue. Vpr colocalization (yellow) of Vpr and SMI312 can be seen in the far right column for both the basal ganglia (**BG**) and frontal cortex (**FC**) of this patient. No primary antibody negative controls are also shown (**Bottom Row**). Pictures were taken at 600x magnification

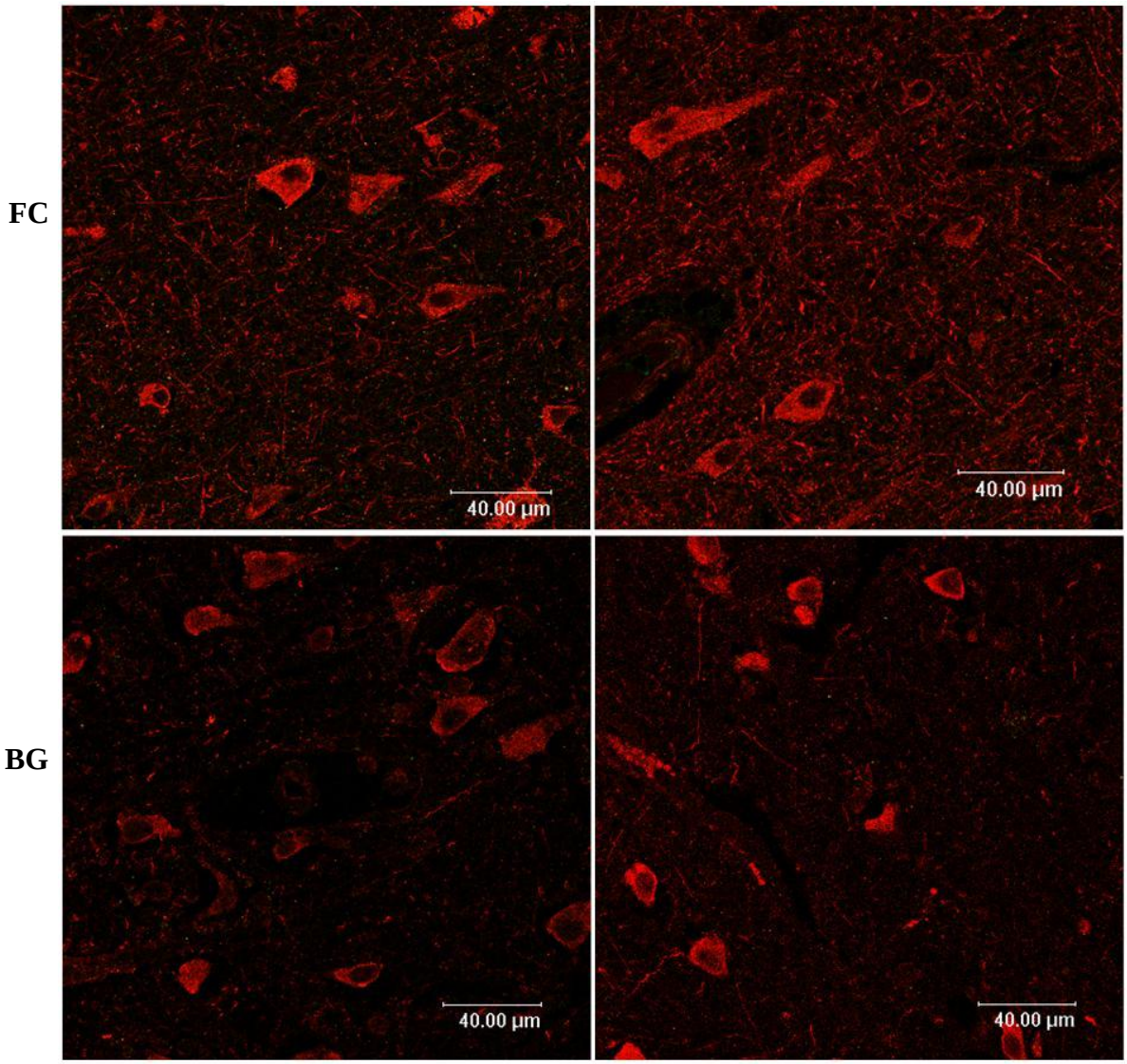

Figure 17: HIV seronegative controls for Vpr and SMI312 double label immunohistochemistry

HIV seronegative controls were incubated with a mixture of a rabbit polyclonal Vpr specific antibody (green) and a mouse monoclonal SMI312 neuron specific antibody (red). Seronegative patients displayed no Vpr positive staining in either the basal ganglia or frontal cortex (representative of 4 seronegative patients for both the basal ganglia and frontal cortex). Pictures were taken at 600x magnification.

5. DISCUSSION

HIV-1 Associated Dementia (HAD) is the most severe neurological complication associated with HIV-1 infection (25). The most common pathological features associated with HAD include neuronal inflammation and neurodegeneration, which are collectively known as HIV Encephalitis (HIVE) (26). While the occurrence of HAD is on the decline since the introduction of HAART, the prevalence of HIVE is rising. This increase in HIVE is at least in part due to the increased survival times of patients (26). Treatment with HAART is able to decrease plasma viral loads, therefore decreasing the amount of HIV-1 traveling to the brain, resulting in a reduction in the occurrence of the severe end-stage HAD. However, due to the increased survival times of patients on HAART, enough HIV-1 is able to enter the brain over time, resulting in the neuronal damage associated with HIVE. One additional contributing factor to the rise in HIVE prevalence is that even in the presence of HAART, the CNS remains relatively isolated from systemic circulation. This isolation allows the latent or slowly replicating viruses present in the CNS to be protected from HAART (18). Another contributing factor to the rise of HIVE prevalence is that HAART is not available to 90 percent of HIV infected individuals due to cost concerns (49). The overall effect of these factors results in an increase in the incidence of HIVE.

The neuronal damage and loss seen during HIVE has been shown to be predominantly due to apoptosis (34). The neurotoxic effects are thought to at least in part be caused by HIV-1 viral proteins interacting with the neurons and causing neuronal damage or death (31-33). Several HIV-1 viral proteins are associated with the neuronal apoptosis seen in HIVE including

gp120, Tat, Nef, and Vpr (51, 53, 54, 64). *In vitro* analyses indicate that HIV-1 Vpr causes neuronal apoptosis using several apoptotic pathways (54, 83, 89, 90). Despite the suggested roles of Vpr in HIV neuropathogenesis (115), no one has shown *in vivo* the presence of Vpr in HIVE brain tissue. The overall objective of my project was to [1] determine the presence of detectable amounts of Vpr in the brain tissue of HIVE patients, and [2] determine which cell types present in the HIVE brain tissue were positive for Vpr.

To address this question, a panel of eight HIVE patients and four HIV seronegative patients was created. Tissue section from both the basal ganglia and frontal cortex were used in this study because they are the sites of the brain most heavily infected with HIV during HIVE (34). One of the most abundant antigens detected during HIV infection is p24 (96). Since p24 has previously been shown to be present in HIVE brain tissue (97), the tissue sections in this study were first tested for p24 to confirm the presence of HIV infection. All eight HIVE patients displayed positive p24 staining by immunohistochemistry in the basal ganglia and frontal cortex. These findings corroborated the presence of HIV infection in the brain tissue of the HIVE patients used in this study. These results are also indicative of the presence of actively replicating HIV-1 virus in the brain macrophages of the HIVE patients. If the p24 present in the macrophages was only derived from the presence of the virion in the cell and not active HIV-1 replication, then the amount of p24 in the macrophages would be below the detection limits of the non amplifying immunohistochemistry technique followed for the tissue sections presented in Figure 1. Therefore, based on the high amounts of p24 staining per cell seen in the cytoplasm of the macrophages in Figure 1 (A, B, E, and F), it can be concluded that active HIV-1 viral replication is occurring in these cells.

Based on the results from the p24 staining described above, it can be anticipated that active HIV-1 viral replication will produce accessory proteins in measurable quantities as well. HIV-1 accessory protein Vpr has been shown to be present in detectable amounts in the CSF of HIV infected individuals (88). Vpr has also been shown to be able to transduce into cells not normally infected by HIV (72). In addition, Vpr has been shown to have an apoptotic effect on both astrocytes and neurons (83, 90, 91). In this study, using immunohistochemistry, all eight HIVE patients were shown to have Vpr positive staining in both the basal ganglia and frontal cortex. These findings further support the role of Vpr in neuropathogenesis as suggested by previous publications. Poon *et. al.* (116) demonstrated the extracellular Vpr is present in high enough concentrations to perform biological functions once transduced into a cell *in vitro*. By showing that Vpr is present in large enough quantities to be detected by immunohistochemistry without amplification, it suggests that Vpr is also present in high enough quantities to perform its suggested neurotoxic biological functions *in vivo* within neurons. These results for the first time provide *in vivo* support for the role of Vpr in neuropathogenesis. Though the present study focuses solely on Vpr, it is possible that other HIV-1 viral proteins might also play a role in conjunction with Vpr to cause the neurotoxic effect seen in HIVE.

Within the CNS, several cell types have been shown to be susceptible to HIV-1 infection including macrophages and astrocytes (28, 30). Macrophages have also been shown to be capable of supporting a productive HIV infection during HIVE (29). In this study, the HIVE patients were tested by double label immunohistochemistry to determine if Vpr was present in the macrophages of the encephalitic brain tissue. All the HIVE patients tested displayed positive Vpr staining in their brain macrophages. In addition, the HIVE patients presented an increased number of infiltrating macrophages when compared to HIV seronegative controls. These

findings coincide with the previously published literature describing the activation and accumulation of macrophages around blood vessels during HIVE (97).

Astrocytes play a significant role in brain homeostasis by establishing contact with the brain microvascular endothelial cells, and regulating the permeability of the blood-brain barrier (18). While astrocytes have been shown to be susceptible to HIV infection, this finding was detectable only by sensitive techniques that detect HIV-1 RNA or proviral DNA (92). This may in part explain the astrocytic findings displayed in this study. The HIVE brain tissue was subjected to double label immunohistochemistry to determine if Vpr was present in detectable amounts in the astrocytes of the HIVE patients. None of the HIVE patients tested in this study displayed positive Vpr staining in their astrocytes. This could be due to the sensitivity of the assay used to test the presence of Vpr. During the single label immunohistochemistry for Vpr, some positive staining appeared to be present in cells resembling astrocytes based on their morphology. The double label immunohistochemistry by immunofluorescence, used to test for 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, used to detect the presence of Vpr in the HIVE brain tissue. The findings of this study, along with the low levels of HIV-1 infection shown to be present in astrocytes from previous studies, suggest that Vpr is not present in the astrocytes of HIVE brain tissue, or at least not present in detectable levels. The HIVE patients in this study did however display increased numbers of activated astrocytes surrounding their blood vessels when compared to HIV seronegative controls. This finding supports previous studies showing that during HIVE the metabolic activity of astrocytes increases, and the astrocytes themselves rapidly proliferate in a process known as astrogliosis (112).

During the course of HIVE, the brain undergoes both significant neuronal damage and loss (113). This neuronal loss and damage has been shown to be caused predominantly by apoptosis (34). Neuronal apoptosis seen in HIVE can be induced directly by exposure to HIV-1 viral proteins, or indirectly by exposure to cytokines and chemokines released from HIV-1 infected brain macrophages (117, 118). While a controversy still remains about whether neurons can be infected by HIV or not (111, 119), the most popular view is that neurons are not susceptible to infection by HIV. Neurons have however been shown to be susceptible to HIV-1 viral proteins acting as neurotoxins during HIVE (18, 31-33). Specifically, Vpr has been shown to be capable of being taken up by non-proliferating cells including neurons (114). In this study HIVE patients were tested by double label immunohistochemistry to determine if Vpr was present in detectable amounts in the neurons of the basal ganglia and frontal cortex. All the HIVE patients displayed Vpr positive staining in a small population of the neurons present in their brain tissue. Showing the presence of Vpr in detectable amounts *in vivo* in the neurons of HIVE brain tissue further supports the neuropathogenic role of Vpr at least in part.

Previous studies from several laboratories have suggested a specific role for Vpr in the neuropathogenesis seen with HIVE, which is the induction of neuronal apoptosis. Vpr has been shown to be capable of inducing apoptosis of neurons through a caspase 8 dependent mechanism (54). Vpr has also been shown to be capable of upregulating the pro-apoptotic Bcl-2 associated death promoter (Bad)(83), which leads to neuronal apoptosis. Additionally, through interactions with its amino terminal region, Vpr can form ion channels across planar lipid bilayers of neuronal cellular membranes, disrupting the ionic gradient of the neuronal cells (89, 90). This disruption causes a large inward cation current and depolarization of the neuronal plasmalemma, leading to neuronal cell death. Combined, these studies provide ample *in vitro* evidence for the

role of Vpr in the induction of neuronal apoptosis. While a role has been suggested *in vitro* for Vpr in neuronal apoptosis, prior to this study Vpr protein had not been shown *in vivo* in the brain tissue of HIV infected patients. These findings support the role of Vpr specifically on neuronal apoptosis by showing the presence of Vpr *in vivo* in neurons at high enough levels to induce apoptosis.

Based on the findings of this study, in conjunction with the findings of previous studies on the neurotoxic effects of Vpr, I have proposed a model suggesting how Vpr in part could play a role in neuropathogenesis during HIVE (Figure 18). It has been previously proposed that HIV enters the through what has become known as the "Trojan horse" hypothesis (27). This is when infected monocytes cross the blood-brain barrier to replenish the perivascular macrophage population. Once the infected monocyte has differentiated into a macrophage, it is capable of supporting a productive HIV infection (120). HIV virions can then be released from the macrophages and infect astrocytes (92). Alternatively, HIV infected macrophages can release neurotoxic effects that directly damage or cause death to neurons (121). In addition, HIV-1 viral proteins can be released by infected macrophages during HIVE (31-33). With previous findings in conjunction with the findings of this study, I propose that Vpr is one of the proteins released from infected macrophages. Once released, the free Vpr can transduce into the neurons in brain parenchyma. After the Vpr has been taken up by the neurons, Vpr can induce apoptosis (83, 90), leading to the neuronal damage and death associated with HIVE.

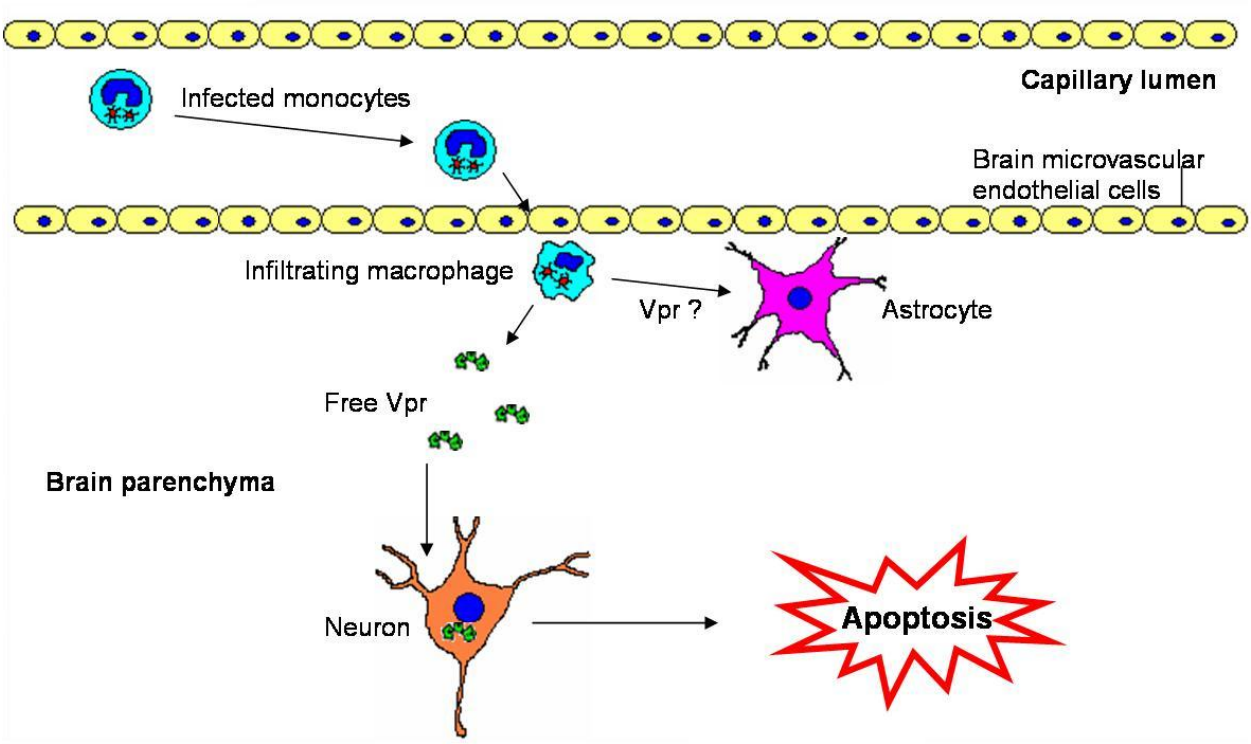

Figure 18: Proposed model for Vpr pathway during HIVE

HIV is thought to enter the brain in infected monocytes that cross the blood-brain barrier (brain microvascular endothelial cells) in what has become known as the "Trojan horse" hypothesis. After reaching the brain parenchyma, the infected monocytes differentiate into perivascular macrophages. These infected macrophages can support productive infection, releasing HIV virions to nearby macrophages and astrocytes. Additionally, the infected macrophages release HIV-1 viral proteins, including Vpr. Free Vpr can then transduce into neurons, and induce apoptosis. This Vpr induced apoptosis in part contributes to the neuronal damage and loss seen in HIVE.

In conclusion, this study for the first time shows 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, of the basal ganglia and frontal cortex of HIVE brain tissue. 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 the HIVE brain tissue, we have created the start of a pathway on which to build on to further understand Vpr induced neurodegeneration. Further understanding of role of Vpr in neuropathogenesis could lead to the development of therapeutic treatments that could either reduce or prevent the HIV public health epidemic.

6. FUTURE DIRECTIONS

In future studies, the apoptotic pathways employed by Vpr to induce neuronal apoptosis suggested by *in vitro* studies should be confirmed *in vivo* in the presence of HIVE. To do this, brain tissue section from HIVE patients can be subjected to triple label immunohistochemistry to determine the presence of apoptotic markers in the same neurons positive for Vpr. Vpr has been shown to induce apoptosis through both a caspase 8 dependent mechanism, and a Bad dependent mechanism. Annexin V is an early stage marker of apoptosis during caspase activation induced apoptosis. The presence of annexin V in those neurons positive for Vpr should be determined to preliminarily confirm the use of the caspase 8 dependent mechanism of apoptosis by Vpr during HIVE. Triple label immunohistochemistry on brain tissue from HIVE patients using Vpr, active caspase 8, and SMI312 (neuronal) markers can also be used to determine the presence of increased levels of active caspase 8 in those neurons positive for Vpr. A mitochondrial membrane sensor technique also exists to show the changes in the mitochondrial membrane potential occurring during the mitochondrial pathway of apoptosis. The detection of a change in the mitochondrial membrane potential should be determined in those neurons positive in HIVE brain tissue. Triple label immunohistochemistry using Vpr, Bad, and SMI312 (neuronal) markers can also be used to determine the presence of increased levels of Bad in those neurons positive for Vpr in HIVE brain tissue.

Another future study based on the findings of this study would be to confirm that free Vpr released by brain macrophages is taken up by neurons, causing a neurotoxic effect. A transwell system can be used, in order to address this question. The transwell insert would

contain blood derived HIV-1infected monocytes differentiated into macrophages from patients infected with HIV. A microporous membrane would be present below the HIV-1 infected macrophages, allowing the release of free Vpr to the lower chamber of the transwell system. The lower chamber would contain neuronal cells that the free Vpr could transduce into. Immunofluorescent flow cytometric analysis can then be used to determine the presence of Vpr in the neuronal cells from the lower chamber of the transwell plate. This would help support the theory that brain macrophages release free Vpr, which can then transduce into neurons, and cause a neurotoxic effect. Together these studies will further support the role of Vpr in the neuropathogenesis involved with HIVE.

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