Journal of NeuroVirology, 11: 180–189, 2005

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280590922829

Taylor & Francis
Taylor & Francis Group

The central nervous system is a viral reservoir in simian immunodeficiency virus–infected macaques on combined antiretroviral therapy: A model for human immunodeficiency virus patients on highly active antiretroviral therapy

Janice E Clements, Ming Li, Lucio Gama, Brandon Bullock, Lucy M Carruth, Joseph L Mankowski, and M Christine Zink

Retrovirus Laboratory, Department of Comparative Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

This study used a simian immunodeficiency virus (SIV)-macaque model to determine whether virus persists in the central nervous system (CNS) of human immunodeficiency virus (HIV)-infected individuals in which plasma viral load has been suppressed by highly active antiretroviral therapy. SIV-infected macaques were treated with two reverse transcriptase inhibitors: PMPA (9-*R*-(2-phosphonomethoxypropyl)adenine), which does not cross the blood-brain barrier, and FTC (beta-2′,3′-dideoxy-3′-thia-5-fluorocytidine), which does. Viral DNA and RNA were quantitated in the brain after 6 months of suppression of virus replication in blood and cerebrospinal fluid (CSF). Viral DNA was detected in brain from all macaques, including those in which peripheral viral replication had been suppressed either by antiretroviral therapy or host immune responses. Significant neurological lesions were observed only in one untreated macaque that had active virus replication in the CNS. Expression of the inflammatory markers, major histocompatibility complex (MHC) II and CD68 was significantly lower in macaques treated with PMPA/FTC. Thus, although antiretroviral treatment may suppress virus replication in the periphery and the brain and reduce CNS inflammation, viral DNA persists in the brain despite treatment. This suggests that the brain may serve as a long-term viral reservoir in HIV-infected individuals treated with antiretroviral drugs that suppress virus replication. *Journal of NeuroVirology* (2005) **11,** 180–189.

Keywords: antiretroviral; HIV; reservoir; SIV

Introduction

Antiretroviral therapy for human immunodeficiency virus (HIV)-infected individuals currently consists of a combination of three or more drugs, including nucleoside and non-nucleoside reverse transcriptase, protease, and fusion inhibitors. Despite the effectiveness of highly active antiretroviral therapy (HAART) in controlling virus replication in the peripheral blood, latent reservoirs of replication-competent virus exist in resting $CD4^+$ lymphocytes in the peripheral blood (Chun *et al*, 1997b; Finzi *et al*, 1997; Wong *et al*, 1997). This reservoir is highly stable, with a half-life of 44 months measured in both adults and children who maintained control of viral load in plasma on HAART (Chun *et al*, 1997a; Finzi *et al*, 1999; Zhang *et al*, 1999). Analyses of the

Address correspondence to Janice E. Clements, 733 N. Broadway, Broadway Research Building, 819, Baltimore, MD 21205, USA. E-mail: jclement@jhmi.edu

The authors would like to thank Dr. Sheila Barber for critical comments and Tauni Voelker for expert assistance. This work was supported by grants to JEC (MH07036, NS047984) and MCZ (NS44815, MH069116).

Received 4 October 2004; revised 20 November 2004; accepted 22 November 2004.

viral reservoir in resting $CD4^+$ T cells have confirmed the need for long-term HAART therapy. Evidence is now emerging that additional viral reservoirs, including the central nervous system (CNS), exist in HIV-infected individuals.

Antiretroviral drug combinations have highly variable effects on viral load in the cerebrospinal fluid (CSF) (Cinque *et al*, 2000, 2001; Ellis *et al*, 2000; Gisolf *et al*, 2000; Staprans *et al*, 1999). The blood-brain barrier (BBB) limits the penetration of many antiretroviral drugs into the CNS and for those that do penetrate, it is unclear whether effective drug levels are achieved in the brain (Glynn and Yazdanian, 1998; Kim *et al*, 1998). Autopsy reports of HIV-infected individuals during the last 15 years found no decline in the incidence of inflammatory lesions in the CNS after 1996, when HAART became widely prescribed for HIV-infected individuals (Jellinger *et al*, 2000; Masliah *et al*, 2000). In addition, indirect evidence for the reduced effectiveness of HAART in the brain comes from neuropathological and clinical studies (see Dore *et al*, 1999; Gray *et al*, 2003; Langford *et al*, 2003; Neuenburg *et al*, 2002). These unexpected results suggest that although HAART has been effective in controlling virus load in the peripheral blood, it is less effective in the CNS, and HIV may persist in tissues that do not have effective levels of drug or contain virus-infected cells such as macrophages that are more resistant to the effects of the drugs, particularly the CNS.

The brain is a likely sanctuary for HIV, which may exist as nonreplicating DNA or may undergo periodic replication in response to local activation of infected cells. The brain is a unique reservoir because whereas the BBB restricts the entry of many antiretroviral drugs, activated lymphocytes and monocytes traffic through the brain and carry virus to this sequestered site. Once in the brain, HIV strains that replicate in macrophages can spread to microglial cells that have a slow turnover rate (Hickey, 2001), producing a self-renewing viral reservoir in the CNS. Brain macrophages (some of which are infected) reenter the peripheral blood after entering the meninges, draining from the there to subcutaneous tissues of the head in channels around cranial nerves, entering afferent lymphatics, and passing through lymph nodes and then to peripheral blood (Cserr and Knopf, 1992). This provides a likely route by which CNS viruses, potentially infected with drug resistant viral strains that have been selected in the brain due to low concentrations of antiretroviral drugs in the brain, may reenter the periphery.

Recent studies have demonstrated that virus persists in peripheral blood monocytes in patients on HAART, with a similar half-life to virus in resting $CD4^+$ T cells (Crowe and Sonza, 2000; Furtado *et al*, 1999; Lambotte *et al*, 2000; Sonza and Crowe, 2001; Sonza *et al*, 2001; Zhu *et al*, 2002). Because the estimated mean life span of monocyte-macrophages in blood and peripheral tissues is estimated to be 14 days, this suggests that the viral reservoir in monocytes is renewed from precursor cells in the bone marrow or by ongoing transmission of virus from infected to uninfected monocytes. Further, peripheral blood monocytes traffic into a variety of tissues, including brain, lung, and lymph nodes where they differentiate into macrophages and remain for months, potentially transmitting virus to other cells, thereby perpetuating infection. It is difficult to sequentially sample tissues in HIV-infected humans; however, the role of tissue macrophages as reservoirs for virus can be examined in the simian immunodeficiency virus (SIV) model.

SIV-macaque models of acquired immunodeficiency syndrome (AIDS) are well established and have been extremely useful in studying HIV-1 vaccine development and the pathogenesis of AIDS. However, they have not been widely utilized for the study of HIV-1 infection under HAART because of limited inhibition of the SIV protease by HIV protease inhibitors. To study whether the brain serves as a viral reservoir in the face of potent antiretroviral therapy, we developed an SIV-macaque model of HIV-infected individuals on HAART. We inoculated them with a neurovirulent molecular clone of SIV, SIV/17E-Fr, that has been shown to replicate in the brain and cause CNS lesions (Flaherty *et al*, 1997; Mankowski *et al*, 1997) and treated them with a combination of PMPA (9-***R***-(2-phosphonomethoxypropyl)adenine), a reverse transcriptase inhibitor that does not cross the BBB (Balzarini *et al*, 1990; Fox *et al*, 2000) and FTC (beta-2′,3′-dideoxy-3′-thia-5-fluorocytidine), which does (Schinazi *et al*, 1992). This treatment is typical of the combination drug regiment used in HIV-infected individuals that contains a single CNS-penetrating drug (such as stavudine, zidovidine, abacavir, efavirenz, nevirapine, and indinavir). The use of FTC is timely as this drug was approved in 2003 by the Food and Drug Administration for use in HIV-infected individuals. This combination of antiretroviral drugs effectively suppressed viral load in the peripheral blood for 6 months, although latent SIV was detected in the resting $CD4^+$ lymphocytes in peripheral blood, lymph nodes, and the spleen (Shen *et al*, 2003).

The SIV-macaque model of HIV HAART was used in this study to determine the ability of antiretroviral drugs that control virus replication in the peripheral blood to suppress virus replication in the CNS. Viral load in plasma and CSF was examined longitudinally during infection, and virus replication, inflammatory markers, and neuropathology were quantitated in the CNS to determine the effects of antiretroviral drugs on virus replication and host inflammatory responses in the brains. Four macaques were inoculated with SIV/17E-Fr and treatment with PMPA/FTC was initiated in two of the macaques at 6 weeks post inoculation (p.i.). Virus was detected in the plasma and CSF by 7 days after inoculation. Once antiretroviral

therapy was initiated, virus replication in the peripheral blood and CSF declined, although it was occasionally detected in the treated macaques and more so in the untreated macaques. The macaques were euthanized after 6 months of treatment and viral RNA and DNA levels were quantitated in three regions of the brain by real-time polymerase chain reaction (PCR).

Viral RNA was undetectable in the brains of the two treated macaques and in one of the untreated macaques. However, viral DNA was found at similar levels in the brains of all four macaques, including the three macaques without active viral replication. Significant neurological lesions were observed only in a single untreated macaque with active virus replication in the CNS. Expression of the macrophage markers major histocompatibility complex (MHC) II and CD68 was significantly lower in macaques that were treated with PMPA/FTC than in untreated controls. Thus, antiretroviral treatments that penetrate the CNS may play a dual role in suppressing viral replication in the periphery and the brain and reducing CNS inflammation. Further, the brain serves as a long-term virus reservoir in SIV-infected macaques treated with antiretroviral drugs that suppress virus replication in the peripheral blood and cross the BBB.

Results

Combined antiretroviral therapy suppressed viral load in plasma and CSF

Four pigtailed macaques were inoculated intravenously with SIV/17E-Fr, a macrophage-tropic, neurovirulent infectious molecular clone (Flaherty *et al*, 1997; Mankowski *et al*, 1997). Virus in peripheral blood and CSF of all macaques was detectable at 1 week p.i. and peaked at 2 weeks p.i. (Figure 1). The viral load in plasma has been reported (Shen *et al*, 2003) and is presented here for comparison with viral load in the CSF. Viral load in the CSF at 2 weeks p.i. was 2 logs lower than in the peripheral blood (Figure 1); this also has been observed in an accelerated, consistent model of SIV AIDS and CNS disease (Zink *et al*, 1999). Viral loads in the peripheral blood of one HAART-treated and one untreated macaque (98P008 and 98P009) were 2 logs higher during acute infection than in macaques 9898P004 and 9897P021. Macaques 98P008 and 98P009 also

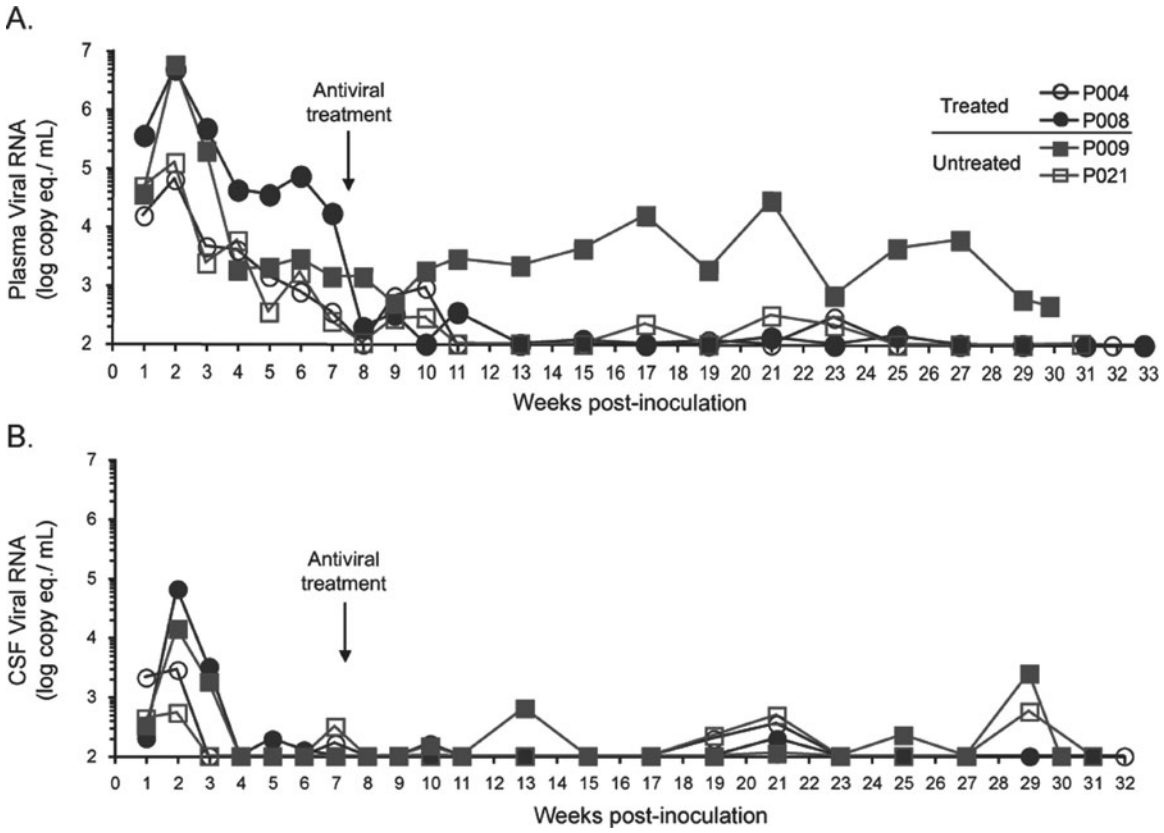

Figure 1 Viral RNA in CSF and plasma from SIV-infected macaques treated with combined antiretroviral therapy. (**A**) Plasma viral load was measured longitudinally by real-time RT-PCR (sensitivity 100 copies/ml). Treatment with PMPA and FTC was initiated at 50 days p.i. (**B**) CSF viral RNA measured longitudinally by real-time RT-PCR. Solid lines indicate macaques that were treated with antiretroviral drugs. Solid symbols indicate macaques that had high peak plasma viral RNA levels during acute infection.

had higher viral load in the CSF (0.75 to 1.25 logs) at 2 weeks p.i. Viral load in the CSF decreased to undetectable or low levels by 4 weeks p.i., after which it was only sporadically detected in the CSF. In comparison, 3 to 5 logs of virus was present in plasma at 4 weeks p.i.

PMPA and FTC treatment was initiated at 50 days p.i. once the viral set point was established in the plasma. Two macaques, 98P004 with low peak plasma viral load and 98P008 with high peak plasma viral load, were treated with PMPA/FTC subcutaneously once daily. The other two macaques (98P009 and P0021) were treated with saline subcutaneously on the same schedule. Viral load in plasma was suppressed by antiretroviral therapy in the two treated macaques and by host immune responses in the untreated macaque that had lower peak plasma viral load (97P021). Viral load was very low in the CSF of all four infected macaques prior to treatment. After the initiation of therapy, plasma viral load in the treated macaques declined; 12 weeks p.i. it was continuously below our level of detection (100 copy equivalents/ml), with the exception of one or two brief periods of breakthrough between 21 and 25 weeks p.i. Viral load in the plasma of the untreated macaque that controlled virus replication naturally (97P021) followed the same pattern except that it was above our level of detection three times 12 weeks p.i. In contrast, plasma viral load in the untreated macaque (98P009) that did not control virus replication naturally remained detectable at 2 to 3 logs throughout infection.

Viral load in the CSF of the two treated macaques was continuously below the level of detection 12 weeks p.i., with the exception of a single elevation at 21 weeks p.i. in both animals. In contrast, viral load in the CSF of the two untreated macaques was detected at higher levels sporadically throughout infection. It is important to note that when virus was detectable in the CSF of the PMPA/FTC-treated macaques, 98P004 and 98P008, at 21 weeks p.i. and in the untreated macaque 97P021 at 29 weeks p.i., there was no detectable virus in the plasma. This suggests that virus in the CNS replicates independently of that in the plasma and that activation of virus replication in the brain can occur while virus replication is suppressed in the peripheral blood by antiretroviral therapy.

In the three macaques that controlled virus replication in the plasma, virus replication in CNS measured by viral load in the CSF was likely due to replication of virus in a small number of persistently infected cells or reactivation of virus in cells that were latently infected. Because plasma viral load was below the level of detection in these animals, it is unlikely that infected cells from the peripheral blood were entering the CNS and replicating virus in brain. The untreated macaque 98P009 that had persistent virus detectable in the plasma throughout the study had the lowest $CD4^+$ lymphocyte counts (<200 cells/μl) terminally. Thus, virus in the CSF of this macaque might have been due to entry of infected cells from the periphery but more likely from persistently or latently infected cells activated in the brain.

Combined antiretroviral therapy suppressed RNA but not DNA levels in brain

To determine whether there was virus replication or evidence of latent virus in the brain in the SIV-infected macaques in this study, viral RNA and DNA levels in the brain were quantitated by real-time PCR in three regions of the brain (basal ganglia, parietal cortex, and thalamus). Viral replication, measured by the presence of full-length viral RNA, was detected in all three regions of the brain of 98P009: basal ganglia (255 copy equivalents/μg RNA), parietal cortex (26,343 copy equivalents/μg RNA), and thalamus (4989 copy equivalents/μg RNA). In contrast, there was no detectable viral RNA in the brains of the other three macaques.

In contrast to viral RNA, viral DNA was detected at significant levels in all three brain regions in all macaques, both treated and untreated (Figure 2). Further, all three of the macaques that suppressed viral load in plasma, either naturally or as a result of antiretroviral therapy, had similar levels of viral DNA, suggesting that neither immune responses or antiretroviral treatment impacted the number of copies of viral DNA in the brain. There were no significant differences in the level of viral DNA among the four macaques, although the level of viral DNA in the brain of macaque P009 tended to be higher. These data suggest that viral DNA persists in the brain for months in the absence of replicating virus in the peripheral blood. Because the brain is sequestered from

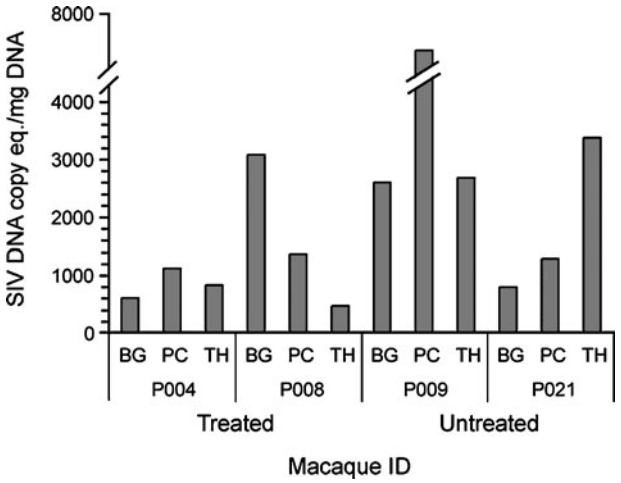

Figure 2 SIV viral DNA in three regions of the brain of infected macaques treated/untreated with PMPA and FTC. SIV DNA was quantitated in basal ganglia (BG), parietal cortex (PC), and thalamus (TH) by real time PCR. The mean level for each region is shown. The mean levels of DNA were not statistically different between the macaques in which virus replication in the plasma was suppressed (98P004, 98P008, and 97P021) and the macaque that did not control plasma virus replication (98P009).

the periphery by the BBB, it may constitute a unique viral reservoir during HAART therapy.

MCP-1 expression was suppressed in macaques that controlled CNS virus replication

The chemokine macrophage chemoattractant protein (MCP)-1 plays a critical role in the entry of monocytes into the brain. Previous studies from our laboratory using the SIV model as well as studies of HIV-infected individuals have indicated that MCP-1 levels in the CSF may be prognostic indicators for the development of SIV/HIV CNS disease (Zink *et al*, 2001; Kelder *et al*, 1998). We examined the effect of combined antiretroviral treatment on the levels of MCP-1 in the CSF and plasma of treated and untreated SIV-infected macaques.

CSF MCP-1 levels rose in all animals during acute infection, a pattern typical of SIV infection (Zink *et al*, 2001). After initiation of antiretroviral therapy, CSF MCP-1 levels declined and remained at preinoculation levels in the treated macaques. The maintenance of steady-state levels of MCP-1 in the CSF of the two treated macaques and the untreated macaque that controlled virus replication naturally suggest that suppression of virus replication in the brain results in reduced synthesis of MCP-1 in the brain. There were periodic increases in CSF MCP-1 in the untreated macaques. In particular, macaque 98P009 had substantially elevated MCP-1 levels in CSF during late infection, consistent with the pattern we see in macaques with SIV encephalitis. The relatively high level of MCP-1 in the CSF of macaque 98P009 (750-1000 pg/ml at 26 to 29 weeks p.i.) (Figure 3) suggests active inflammatory processes in the brain.

Combined antiretroviral treatment or natural suppression of viral replication reduces severity of CNS lesions

Only the untreated macaque (9898P009) that did not naturally suppress virus replication had detectable viral load in plasma throughout the study. This macaque also had significant immunosuppression measured by the number of CD4+ lymphocytes. It is well established that high viral load and immunosuppression frequently accompany or precede the development of CNS lesions in the SIV model (Zink and Clements, 2002; Zink *et al*, 1999, 2001). When the brains of the four macaques were assessed for the presence of SIV encephalitis or expression of viral protein or RNA, only macaque 98P009 had significant encephalitis, which consisted of a moderate number of macrophage- and lymphocyte-rich perivascular cuffs as well as multifocal glial nodules predominantly located in white matter. Lesions were present in many neuroanatomical sites in the brain of this animal including basal ganglia, thalamus, cerebral cortex, and midbrain. There also was substantial expression of the viral protein gp41, particularly in white matter. Two other animals, 98P008 and 97P021, developed equivocal encephalitis with occasional, scattered perivascular lymphocytes, and a small number of macrophages in white matter. However, no expression of SIV gp41 was detected in the brain. Lesions were not present in the remaining animal 98P004; SIV gp41 was not detected in this animal by immunohistochemistry.

Increased expression of inflammation/activation markers in the CNS of macaques with higher peak acute virus replication

Inflammatory/activation markers in macrophages (CD68 and MHC class II) and astrocytes (glial fibrillary acidic protein [GFAP])were quantitated by digital image analysis of immunohistochemically stained brain sections to assess the impact of combined antiretroviral suppression of plasma viral load on inflammation/activation of macrophages in the brain. Inflammatory responses in the CNS may, in part, be a response to activation of inflammatory cytokines and chemokines in the peripheral blood that are produced in response to viral replication. Astrocytes have significantly increased expression of GFAP in response to acute virus replication in the CNS of

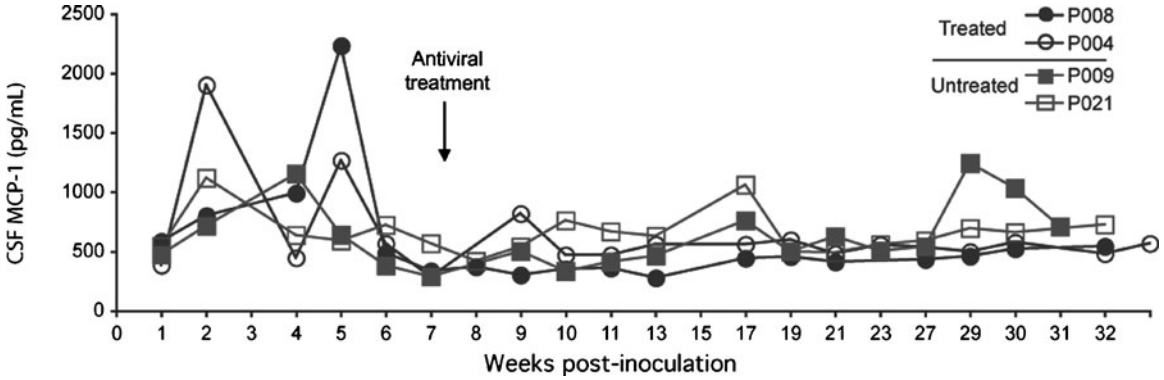

Figure 3 Macrophage chemoattractant protein-1 (MCP-1) in the CSF measured longitudinally in SIV-infected macaques by ELISA. Only macaque 9898P009 had significant increases in CSF MCP-1 after the initiation of treatment.

Table 1 Expression of inflammation/activation markers in the CNS

Animal	*Treatment*	*Peak plasma viral load*	*CNS lesions**	*GFAP*	*CD68*	*MHC II*
98P004	+	Low	0.3	454	171	233
97P021	−	Low	0.2	366	1014	587
				$P = .294$	$P < .001$	$P < .0001$
98P008	+	High	0.6	437	447	210
98P009	−	High	1.5	383	7569	610
				$P = .437$	$P < .001$	$P < .0001$

*Lesion score is out of a maximum of 3, with values from 0 to 0.5 indicative of no encephalitis, from 0.51 to 1.0 representing mild encephalitis, from 1.1 to 2 representing moderate encephalitis, and 2.1 to 3 representing severe encephalitis.

SIV-infected macaque by 21 days p.i. (Mankowski *et al*, 2002). In this study, in which antiretroviral treatment was initiated at 50 days p.i., there was no significant difference observed in the levels of GFAP between macaques receiving combined antiretroviral therapy and placebo (Table 1). In contrast, the untreated macaque with high peak plasma virus load, 98P009, had significantly higher expression of CD68 than the treated macaque with high peak plasma viral load ($P < .001$; Table 1). Similarly, CD68 expression in the macaques with low peak plasma viral load, 97P021 (untreated), was significantly higher than in 98P004, the treated macaque with low peak plasma viral load. The expression of MHC class II revealed similar differences between the treated and untreated macaques in the high (98P008 and 98P009) and low (98P004 and 97P021) virus load groups (Table 1). These data suggest that astrocyte activation is not significantly altered by antiretroviral therapy administered on this time schedule, whereas activation of macrophages in the CNS appears to be sensitive to treatment.

Discussion

Treatment of HIV-infected individuals with HAART controls virus replication in the peripheral blood but the question of the impact of treatment on virus replication or latent virus in the brain has been difficult to assess. In this study we demonstrate for the first time that SIV DNA persists in the brain of infected macaques in which virus replication in the peripheral blood has been suppressed by combined antiretroviral therapy or by host immune responses. Viral DNA persisted in the brain for over 20 weeks after the control of virus replication. Treatment also maintained the suppression of viral load in the CSF that occurred naturally after acute infection. Although PMPA does not have significant penetration into the brain, FTC has been reported in macaques to penetrate the BBB, with drug levels of 2% to 7% of that found in the peripheral blood (Schinazi *et al*, 1992).

We have previously demonstrated that virus replication occurs in the brain as early as 10 days p.i. in SIV-infected macaques. Virus replication in the CNS is down regulated by 21 days p.i. but viral DNA levels are unchanged in the brain at 10, 21, and 56 days p.i. in SIV-infected macaques in which there is ongoing virus replication in the peripheral blood (Clements *et al*, 2002). In this study, virus was detected in the CSF by 7 days p.i., suggesting that the brain was infected within the first week. Viral DNA persisted in the brain for at least 20 weeks p.i. without significant virus replication in the peripheral blood of combined antiretroviral treated animals.

We have previously reported that virus replication in the CNS is controlled independently from that in the periphery. Thus it was not unexpected that viral load decreased to very low levels after peak virus replication. However, it was interesting that virus was detected in the CSF of macaques (98P004 and 98P008) at 19 to 21 weeks whereas there was no detectable virus in the plasma. This suggests that viral DNA genomes in brain are not only stable but also replication competent and that virus can be reactivated in the brain independent of replication in the periphery. This is further supported by the production of infectious virus by microglial cells isolated from the brain of macaque 98P008 (data not shown).

Antiretroviral drugs are less effective in suppressing virus replication in macrophages than in lymphocytes (Crowe *et al*, 1989; Perno *et al*, 1994, 1998). One study showed that a 4- to 10-fold higher concentration of ritonavir and saquinavir was required to completely suppress virus replication in chronically infected macrophages derived from blood as compared to lymphocytes (Perno *et al*, 1998). In addition to brain macrophages and microglia, astrocytes also are infected in the brain of HIV-infected children and adults (Ranki *et al*, 1994; Saito *et al*, 1994; Tornatore *et al*, 1994). Our observation that combined antiretroviral therapy did not change the expression of GFAP in astrocytes suggests that these cells may be activated early during acute infection and remain at the same level of activation even without ongoing virus replication in the peripheral blood or, more surprisingly, in the brain. Astrocytes are the most abundant cell in the brain (ranging from 4 to 20 $\times$ 10^{11} total cells) and the frequency of infected astrocytes has been estimated to be from 0.5% to 2.5% (Brack-Werner, 1999). Astrocytes are critical cells in the CNS, providing homeostatic functions for inflammatory responses as well as supporting the integrity of the BBB. HIV and SIV infection of astrocytes *in vivo* and *in vitro* is noncytopathic; thus, the virus can reside in these cells indefinitely.

In contrast to astrocytes, expression of the inflammatory markers CD68 and MHC class II on macrophages in the brain was significantly lower in macaques treated with combined antiretroviral drugs

as compared to the untreated macaques, despite the fact that one of the latter had no detectable viral RNA in the brain. In macaques 98P004 and 98P008 the level of expression of the macrophage activation markers was significantly reduced as compared to the untreated macaques. This suggested that combined antiretroviral treatment not only suppressed virus replication but also impacted factors that promote activation and inflammation of macrophages in the brain. This may explain how antiretroviral drugs that enter the CNS as well as those that do not penetrate the CNS may alter the severity of neurological disease in humans infected with HIV.

The BBB limits access of most of the antiretroviral drugs used to control HIV replication in HAART (Glynn and Yazdanian, 1998; Sawchuk and Yang, 1999). Effective levels of drugs are not usually achieved in the brain and a number of drugs that do enter the brain are actively removed by drug transporters that actively pump drug out of the brain (Kim *et al*, 1998). The persistence of viral DNA in the brain in this study suggests that even when antiretroviral drugs enter the CNS, the brain remains a significant reservoir for latent virus. The finding of brief periods of increased CSF viral RNA in the absence of increased viral load in the plasma suggests that activation of virus replication likely occurs during the course of asymptomatic infection. Activation of virus replication in the brain has the potential for introducing these strains into the peripheral blood and increasing the burden of drug resistant viruses. Because the CNS has lower effective levels of antiretroviral drugs, there is a higher likelihood that drug resistant virus will be selected. Thus, the brain must be considered in strategies aimed at eliminating latently infected cells from HIV infected individuals.

Materials and methods

Virus

SIV/17E-Fr is a macrophage-tropic and neurovirulent recombinant strain derived from SIVmac239 by substituting the entire *env* and *nef* genes as well as the 3′ long terminal repeats of SIVmac239 with those from SIV/17E-Br, a virus derived from SIVmac239 by serial passage in rhesus macaques and subsequent isolation from the brain of a macaque with fulminant encephalitis (Flaherty *et al*, 1997; Mankowski *et al*, 1997).

Animal experiments

Four juvenile pig-tailed macaques (*Macaca nemestrina*), aged 2 to 3 years, were inoculated intravenously with SIV/17E-Fr (10,000 tissue culture infectious doses [$TCID_{50}$]). Beginning on day 50 p.i., two macaques (98P004 and 98P008) were injected once daily subcutaneously with the antiretroviral drugs PMPA (or tenofovir) and FTC at 20 and 50 mg/kg/day, respectively. Beginning on day 85 p.i., the dose for PMPA was increased to 30 mg/kg/day for the rest of the study. The other two animals (98P009 and 97P021) were administered drug vehicle daily on the same schedule and served as untreated controls. PMPA is a nucleotide reverse transcriptase (RT) inhibitor that does not cross the BBB (Balzarini *et al*, 1990; Fox *et al*, 2000), and FTC is a nucleoside RT inhibitor that crosses the BBB (Schinazi *et al*, 1992).

Blood was drawn from each animal for plasma viral load assay weekly until day 77 p.i. and semimonthly thereafter. The animals were euthanized at 29 to 33 weeks p.i. Prior to necropsy, animals were perfused with sterile phosphate-buffered saline (PBS) to remove virus-containing blood from the tissues.

Plasma and CSF viral load

Virion-associated SIV RNA in plasma and CSF was measured using a real-time RT-PCR assay on an Applied Biosystems Prism 7700 Sequence Detection System (the Taqman method), as previously described (Hirsch *et al*, 1996; Suryanarayana *et al*, 1998). For each sample, three reactions were performed. Duplicate aliquots were separately reverse transcribed and amplified, and the amplification cycle during which a detectable PCR product was first observed (threshold cycle) was determined from real-time kinetic analysis of fluorescent product generation as a consequence of template-specific amplification (Suryanarayana *et al*, 1998). To control for DNA contamination, one reaction was processed and amplified without addition of RT (no RT control). Nominal copy numbers for test samples were then automatically calculated by interpolation of the experimentally determined threshold cycle values onto a regression curve derived from control transcript standards, followed by normalization for the volume of the extracted plasma or CSF specimen.

Quantitation of SIV RNA and DNA in brain

To quantitate viral RNA and DNA in brain tissues, samples from three regions of the brain (basal ganglia, parietal cortex, and thalamus) were snap frozen in liquid nitrogen for RNA or DNA isolation. RNA was isolated from duplicate samples of 50 mg of basal ganglia, thalamus, parietal cortex, and cerebellum using the RNA STAT-60 Kit (Tel-Test, Friendswood, Texas), followed by DNase treatment and final purification with the RNeasy Kit (Qiagen, New Orleans, Louisiana). DNA was isolated from duplicate samples of 50 mg of basal ganglia, thalamus, parietal cortex, and cerebellum using the Fast DNA Kit (Q-Biogene, Carlsbad, California) following the directions of the manufacturer for the Spin protocol. Using 50 mg of brain, consistent recoveries of RNA and DNA were obtained (Zink *et al*, 1999). Quantitation of SIV RNA and DNA from extracted brain tissue was done using SIV *gag* primers, and probe in a real-time RT-PCR assay, as described (Zink *et al*, 1999). Each sample was quantitated in triplicate and the sensitivity

of detection for the real-time RT-PCR technique was 30 copy equivalents of viral RNA/2 μg total brain RNA.

MCP-1 in plasma and CSF
MCP-1 levels in CSF and plasma from the four macaques throughout infection were measured by enzyme-linkedimmunosorbent assay (ELISA) (R&D Systems). To determine whether there was higher MCP-1 expression in the brain than in the plasma, indicating a gradient of chemokine expression that would result in the net influx of macrophages into the brain, MCP-1 levels were expressed as the ratio of MCP-1 in the CSF versus the plasma.

Quantitative immunohistochemical analysis
KP-1 (Dako, Carpenteria, California), which recognizes the macrophage marker CD68, was used to quantitate activation/infiltration of cells of macrophage lineage, including microglia, in the brain parenchyma. Anti-HLA-DP,-DQ,-DR (clone CR3/43; Dako), was used to detect MHC class II expression in the brain as a marker of activation of macrophages/microglia and endothelial cells. A bovine polyclonal antibody to GFAP (Dako) that recognizes human GFAP and which is highly cross-reactive between species was used to detect macaque GFAP. Quantitation of immunohistochemical staining on tissues was performed using digital image analysis as described (Zink *et al*, 1999).

Histopathology
Sections of CNS, including frontal, parietal, temporal, and occipital cortex, basal ganglia, thalamus, midbrain, medulla, cerebellum, and cervical spinal cord were examined microscopically in a blinded fashion by two pathologists (MCZ, JLM). To quantitate the severity of lesions, sections of frontal and parietal cortex, basal ganglia, thalamus, midbrain, and cerebellum were each given numerical scores of 1 (minimal), 2 (moderate), or 3 (severe) using the following semiquantitative system. Sections with more than 30 perivascular macrophage-rich cuffs were given a score of 3, sections with 10 to 30 perivascular cuffs were given a score of 2, and those with less than 10 perivascular cuffs were given a score of 1. The scores for all sections were totaled and divided by 6 (6 regions were graded for each brain) to give a mean score (out of a maximum of 3) for severity of CNS lesions.

Statistical analysis
RNA and DNA levels for each macaque were measured in each brain region and presented as means of the four regions for each animal. Comparison of the mean values by student's two sample *t* test was validated by comparing the median values of the four regions by the Mann-Whitney test. To test for significant differences in expression of CD68, MHC class II, and GFAP in different macaques, the Mann-Whitney test was used. The Mann-Whitney test is a nonparametric test (an advantage of nonparametric tests is that the test results are more robust against violation of the assumptions) that is considerably more powerful (confidence interval is narrower, on the average) for non-normal populations than the two-sample *t* test.

References

Balzarini J, Naesens L, De Clercq E (1990). Anti-retrovirus activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) in vivo increases when it is less frequently administered. *Int J Cancer* **46:** 337–340.

Brack-Werner R (1999). Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *AIDS* **13:** 1–22.

Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF (1997a). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387:** 183–188.

Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, Lloyd AL, Nowak MA, Fauci AS (1997b). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* **94:** 13193–13197.

Cinque P, Bestetti A, Morelli P, Presi S (2000). Molecular analysis of cerebrospinal fluid: potential for the study of HIV-1 infection of the central nervous system. *J NeuroVirol* **6(Suppl 1):** S95–S102.

Cinque P, Presi S, Bestetti A, Pierotti C, Racca S, Boeri E, Morelli P, Carrera P, Ferrari M, Lazzarin A (2001). Effect of genotypic resistance on the virological response to highly active antiretroviral therapy in cerebrospinal fluid. *AIDS Res Hum Retroviruses* **17:** 377–383.

Clements JE, Babas T, Mankowski JL, Suryanarayana K, Piatak M, Jr., Tarwater PM, Lifson JD, Zink MC (2002). The central nervous system as a reservoir for simian immunodeficiency virus (SIV): steady-state levels of SIV DNA in brain from acute through asymptomatic infection. *J Infect Dis* **186:** 905–913.

Crowe SM, McGrath MS, Elbeik T, Kirihara J, Mills J (1989). Comparative assessment of antiretrovirals in human monocyte-macrophages and lymphoid cell lines acutely and chronically infected with the human immunodeficiency virus. *J Med Virol* **29:** 176–180.

Crowe SM, Sonza S (2000). HIV-1 can be recovered from a variety of cells including peripheral blood monocytes of patients receiving highly active antiretroviral therapy: a further obstacle to eradication. *J Leukoc Biol* **68:** 345–350.

Cserr HF, Knopf PM (1992). Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol Today* **13:** 507–512.

Dore GJ, Correll PK, Li Y, Kaldor JM, Cooper DA, Brew BJ (1999). Changes to AIDS dementia complex in the era of highly active antiretroviral therapy. *Aids* **13:** 1249–1253.

Ellis RJ, Gamst AC, Capparelli E, Spector SA, Hsia K, Wolfson T, Abramson I, Grant I, McCutchan JA (2000). Cerebrospinal fluid HIV RNA originates from both local CNS and systemic sources. *Neurology* **54:** 927–936.

Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C, Quinn TC, Chaisson RE, Rosenberg E, Walker B, Gange S, Gallant J, Siliciano RF (1999). Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* **5:** 512–517.

Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman DD, Siliciano RF (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278:** 1295–1300.

Flaherty MT, Hauer DA, Mankowski JL, Zink MC, Clements JE (1997). Molecular and biological characterization of a neurovirulent molecular clone of SIV. *J Virol* **71:** 5790–5798.

Fox HS, Weed MR, Huitron-Resendiz S, Baig J, Horn TF, Dailey PJ, Bischofberger N, Henriksen SJ (2000). Antiviral treatment normalizes neurophysiological but not movement abnormalities in simian immunodeficiency virus-infected monkeys. *J Clin Invest* **106:** 37–45.

Furtado MR, Callaway DS, Phair JP, Kunstman KJ, Stanton JL, Macken CA, Perelson AS, Wolinsky SM (1999). Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. *N Engl J Med* **340:** 1614–1622.

Gisolf EH, Enting RH, Jurriaans S, de Wolf F, van der Ende ME, Hoetelmans RM, Portegies P, Danner SA (2000). Cerebrospinal fluid HIV-1 RNA during treatment with ritonavir/saquinavir or ritonavir/saquinavir/stavudine. *AIDS* **14:** 1583–1589.

Glynn SL, Yazdanian M (1998). In vitro blood-brain barrier permeability of nevirapine compared to other HIV antiretroviral agents. *J Pharm Sci* **87:** 306–310.

Gray F, Chretien F, Vallat-Decouvelaere AV, Scaravilli F (2003). The changing pattern of HIV neuropathology in the HAART era. *J Neuropathol Exp Neurol* **62:** 429–440.

Hickey WF (2001). Basic principles of immunological surveillance of the normal central nervous system. *Glia* **36:** 118–124.

Hirsch VM, Fuerst TR, Sutter G, Carroll MW, Yang LC, Goldstein S, Piatak M, Elkins WR, Alvord WG, Montefiori DC, Moss B, Lifson JD (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J Virol* **70:** 3741–3752.

Jellinger KA, Setinek U, Drlicek M, Bohm G, Steurer A, Lintner F (2000). Neuropathology and general autopsy findings in AIDS during the last 15 years. *Acta Neuropathol (Berl)* **100:** 213–220.

Kelder W, McArthur JC, Nance-Sproson T, McClernon D, Griffin DE (1998). B-chemokines MCP-1 and RANTES are selectively increased in cerebrospinal fluid of patients with human immunodeficiency virus-associated dementia. *Ann Neurol* **44:** 831–835.

Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM, Wilkinson GR (1998). The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J Clin Invest* **101:** 289–294.

Lambotte O, Taoufik Y, de Goer MG, Wallon C, Goujard C, Delfraissy JF (2000). Detection of infectious HIV in circulating monocytes from patients on prolonged highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **23:** 114–119.

Langford TD, Letendre SL, Larrea GJ, Masliah E (2003). Changing patterns in the neuropathogenesis of HIV during the HAART era. *Brain Pathol* **13:** 195–210.

Mankowski JL, Clements JE, Zink MC (2002). Searching for clues: tracking the pathogenesis of human immunodeficiency virus central nervous system disease by use of an accelerated, consistent simian immunodeficiency virus macaque model. *J Infect Dis* **186 (Suppl 2):** S199–S208.

Mankowski JL, Flaherty MT, Spelman JP, Hauer DA, Didier PJ, Martin Amedee A, Murphey-Corb M, Kirstein LM, Munoz A, Clements JE, Zink MC (1997). Pathogenesis of simian immunodeficiency virus encephalitis: Viral determinants of neurovirulence. *J Virol* **71:** 6055–6060.

Masliah E, DeTeresa RM, Mallory ME, Hansen LA (2000). Changes in pathological findings at autopsy in AIDS cases for the last 15 years. *AIDS* **14:** 69–74.

Neuenburg JK, Brodt HR, Herndier BG, Bickel M, Bacchetti P, Price RW, Grant RM, Schlote W (2002). HIV-related neuropathology, 1985 to 1999: rising prevalence of HIV encephalopathy in the era of highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **31:** 171–177.

Perno CF, Aquaro S, Rosenwirth B, Balestra E, Peichl P, Billich A, Villani N, Calio R (1994). In vitro activity of inhibitors of late stages of the replication of HIV in chronically infected macrophages. *J Leukoc Biol* **56:** 381–386.

Perno CF, Newcomb FM, Davis DA, Aquaro S, Humphrey RW, Calio R, Yarchoan R (1998). Relative potency of protease inhibitors in monocytes/macrophages acutely and chronically infected with human immunodeficiency virus. *J Infect Dis* **178:** 413–422.

Ranki A, Lagerstedt A, Ovod V, Aavik E, Krohn KJE (1994). Expression kinetics and subcellular localization of HIV-1 regulatory proteins Nef, Tat and Rev in acutely and chronically infected lymphoid cell lines. *Arch Virol* **139:** 365–378.

Saito Y, Sharer LR, Epstein LG, Michaels J, Mintz M, Louder M, Golding K, Cvetkovich TA, Blumberg BM (1994). Overexpressionof nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous system. *Neurology* **44:** 474–481.

Sawchuk RJ, Yang Z (1999). Investigation of distribution, transport and uptake of anti-HIV drugs to the central nervous system. *Adv Drug Deliv Rev* **39:** 5–31.

Schinazi RF, Boudinot FD, Ibrahim SS, Manning C, McClure HM, Liotta DC (1992). Pharmacokinetics and metabolism of racemic 2′,3′-dideoxy-5-fluoro-3′-thiacytidine in rhesus monkeys. *Antimicrob Agents Chemother* **36:** 2432–2438.

Shen A, Zink MC, Mankowski JL, Chadwick K, Margolick JB, Carruth LM, Li M, Clements JE, Siliciano RF (2003). Resting CD4(+) T Lymphocytes but not thymocytes provide a latent viral reservoir in a simian

immunodeficiency virus-macaca nemestrina model of human immunodeficiency virus type 1-infected patients on highly active antiretroviral therapy. *J Virol* **77:** 4938–4949.

Sonza S, Crowe SM (2001). Reservoirs for HIV infection and their persistence in the face of undetectable viral load. *AIDS Patient Care STDS* **15:** 511–518.

Sonza S, Mutimer HP, Oelrichs R, Jardine D, Harvey K, Dunne A, Purcell DF, Birch C, Crowe SM (2001). Monocytes harbour replication-competent, non-latent HIV-1 in patients on highly active antiretroviral therapy. *AIDS* **15:** 17–22.

Staprans S, Marlowe N, Glidden D, Novakovic-Agopian T, Grant RM, Heyes M, Aweeka F, Deeks S, Price RW (1999). Time course of cerebrospinal fluid responses to antiretroviral therapy: evidence for variable compartmentalization of infection. *AIDS* **13:** 1051–1061.

Suryanarayana K, Wiltrout TA, Vasquez GM, Hirsch VM, Lifson JD (1998). Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. *AIDS Res Hum Retroviruses* **14:** 183–189.

Tornatore C, Chandra R, Berger JR, Major EO (1994). HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* **44:** 481–487.

Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD (1997). Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* **278:** 1291–1295.

Zhang L, Ramratnam B, Tenner-Racz K, He Y, Vesanen M, Lewin S, Talal A, Racz P, Perelson AS, Korber BT, Markowitz M, Ho DD (1999). Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. *N Engl J Med* **340:** 1605–1613.

Zhu T, Muthui D, Holte S, Nickle D, Feng F, Brodie S, Hwangbo Y, Mullins JI, Corey L (2002). Evidence for human immunodeficiency virus type 1 replication in vivo in CD14(+) monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy. *J Virol* **76:** 707–716.

Zink MC, Clements JE (2002). A novel simian immunodeficiency virus model that provides insight into mechanisms of human immunodeficiency virus central nervous system disease. *J NeuroVirol* **8 (Suppl 2):** 42–48.

Zink MC, Coleman GD, Mankowski JL, Adams RJ, Tarwater PM, Fox K, Clements JE (2001). Increased macrophage chemoattractant protein-1 in cerebrospinal fluid precedes and predicts simian immunodeficiency virus encephalitis. *J Infect Dis* **184:** 1015–1021.

Zink MC, Suryanarayana K, Mankowski JL, Shen A, Piatak J, Spelman JP, Carter DL, Adams RJ, Lifson JD, Clements JE (1999). High viral load in the cerebrospinal fluid and brain correlates with severity of simian immunodeficiency virus encephalitis. *J Virol* **73:** 10480–10488.