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A novel simian immunodeficiency virus model that provides insight into mechanisms of human immunodeficiency virus central nervous system disease

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Although highly active antiretroviral therapy (HAART) has reduced the incidence of dementia, a significant proportion of HIV-infected individuals cease therapy because of unacceptable side effects. Further, HAART is not available to the majority of HIV-infected people worldwide. Thus, animal models remain an important means by which to investigate the pathogenesis of HIV-induced CNS disease, particularly events during acute infection. We have developed an accelerated, consistent SIV/macaque model of HIV infection in which over 90 percent of inoculated macaques develop SIV encephalitis and behavioral changes by three months post-inoculation (p.i.). Viral load in the CSF during terminal infection and the ratio of MCP-1 in CSF:plasma are strong predictors of the severity of encephalitis at necropsy. The high incidence of CNS disease in this model provides an opportunity to correlate host and viral events in the CNS during acute infection with the later development of CNS disease. Using this model, we have demonstrated that viral DNA persists in the brain throughout asymptomatic infection despite significant suppression of active virus replication. We have further demonstrated that encephalitis is associated with the selective replication of macrophage-tropic, neurovirulent viral strains, and that recrudescence of virus replication during terminal infection occurs by reactivation of preexisting viral strains in the brain. *Journal of NeuroVirology* (2002) **8(suppl. 2),** 42–48.

Introduction

Highly active antiretroviral therapy (HAART) has been successful in reducing plasma viral load and improving neurocognitive function in many human immunodeficiency virus (HIV)–infected individuals (Ferrando *et al*, 1998; Perelson *et al*, 1997; Sacktor *et al*, 1999). Nonetheless, many questions remain about the long-term effectiveness of HAART therapy in the central nervous system (CNS). For example, there are a number of reports demonstrating no decline in cerebrospinal fluid (CSF) viral loads in HIV-infected individuals treated with antiretroviral therapy, and to date there have been no comprehensive studies that have examined viral load or pathological changes in the brains of treated individuals after death (Cinque *et al*, 2000, 2001; Gisolf *et al*, 2000).

There is reason for concern because most people do not begin antiretroviral therapy until well after acute infection, when the virus may already have entered and established infection in the brain. Further, many antiretroviral therapeutic agents do not cross the blood-brain barrier, so although they may suppress peripheral virus replication, a parallel viral suppression in the CNS may not occur (Acosta *et al*, 1996; Aweeka *et al*, 1999; Kravcik *et al*, 1999).

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In addition, many patients on antiretroviral therapy are forced to discontinue therapy because of drug toxicity, significant side effects, cost, or the development of drug-resistant mutations (Behrens *et al*, 1999; Carr *et al*, 1999; Fortgang *et al*, 1995; Heath *et al*, 2001; Kopp *et al*, 1997; Kotler *et al*, 1999; Miller *et al*, 1998; Mulligan *et al*, 2000; Periard *et al*, 1999; ter Hofstede *et al*, 2000; Thiebaut *et al*, 2000). At this writing, there are no studies that have examined the effects of treatment cessation on virus replication and host responses in the CNS. Finally, the vast majority of HIV-infected individuals in the world do not have access to HAART and thus, the consequences of infection of the CNS may be profound in the future. These cold facts were the driving force behind our desire to develop a simian immunodeficiency virus (SIV)/macaque model in which there was a very high incidence of neurological disease. Such a model is necessary to correlate virus replication and inflammatory/immune responses in the CNS during acute and asymptomatic infection with outcome during terminal infection.

A unique SIV model of accelerated AIDS and encephalitis

Existing SIV/macaque models provided excellent parallels with HIV infection of humans, requiring years to develop acquired immunodeficiency syndrome (AIDS) and associated neurological diseases in only a subset (25%) of infected animals. However, these SIV animal models could not be used to examine the relationship between acute infection in the CNS and the development of CNS disease, because large groups of animals would be required (very costly) and it was not clear which infected animals would develop CNS disease. Our model is unique because it reproduces, in an accelerated fashion, the events that occur naturally during SIV and HIV infections. The accelerated time course is due to a number of factors. First, the macaques are coinfected with a neurovirulent virus that targets the CNS and an immunosuppressive virus swarm that includes lymphocyte-tropic viruses similar to those that evolve in HIV infection and cause rapid progression to AIDS. The neurovirulent virus has been well characterized biologically and molecularly. It causes CNS disease in macaques and requires the chemokine receptor CCR5 but not CD4 for cell entry (Edinger *et al*, 1997a). The model also uses pigtailed macaques because these animals develop more severe and consistent SIV encephalitis than other macaque species (Zink *et al*, 1997). This model demonstrates that both viral and genetic factors clearly contribute to the time course and development of AIDS and HIV/SIV CNS disease.

In an initial study, 11 of 12 pigtailed macaques coinoculated with SIV/17E-Fr and SIV/DeltaB670 developed SIV encephalitis (4 severe, 5 moderate, and 2 mild) by 84 days post infection (p.i.) (Babas *et al*, 2001a; Zink *et al*, 1999). The SIV encephalitis caused by this inoculation protocol paralleled findings in individuals with HIV encephalitis, with perivascular cuffs of macrophages, multinucleated giant cells, and fewer lymphocytes in addition to randomly distributed microglial nodules and diffuse gliosis, with lesions most severe in the subcortical white matter at the level of the basal ganglia and thalamus. There was abundant expression of viral RNA and antigen in macrophages and giant cells throughout the brain.

To determine whether viral load in the periphery correlated with the development and/or severity of encephalitis, plasma viral RNA was quantitated by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) on samples taken every 2 weeks throughout infection (Mankowski *et al*, 2002; Zink *et al*, 1999). Plasma viral load increased in all infected macaques during acute infection, peaking at 10 to 14 days p.i. (Figure 1). After a 0.5 to 1.0 log decline in some animals, plasma viral load rose again by 42 days p.i. and was maintained at 10^7 to 10^9 copy equivalents per milliliter during the last month of infection. There was no relationship between plasma viral loads and the incidence or severity of SIV encephalitis at necropsy (Zink *et al*, 1999).

In contrast, CSF viral load during asymptomatic and terminal infection did correlate with the severity of SIV encephalitis (Zink *et al*, 1999). As with plasma viral load, CSF viral RNA increased in all macaques, peaking at 10 to 14 days p.i., after which it declined in most macaques (Figure 2). During the second month of infection, CSF viral load increased again, with the highest increases in macaques that developed the most severe encephalitis. By 42 days p.i., the level of CSF viral load predicted whether the macaques would have encephalitis at necropsy.

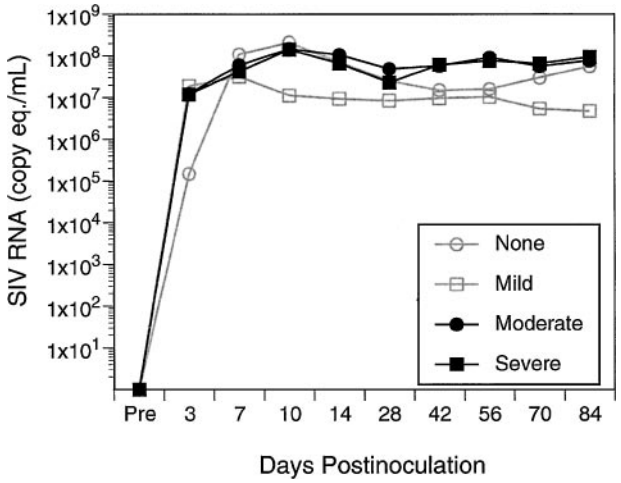

Figure 1 SIV RNA levels in plasma sampled from infected macaques every 2 weeks throughout infection, as determined by real-time RT-PCR. Lines represent mean values for macaques that developed no ($n = 1$), mild ($n = 2$), moderate ($n = 5$), or severe ($n = 4$) encephalitis.

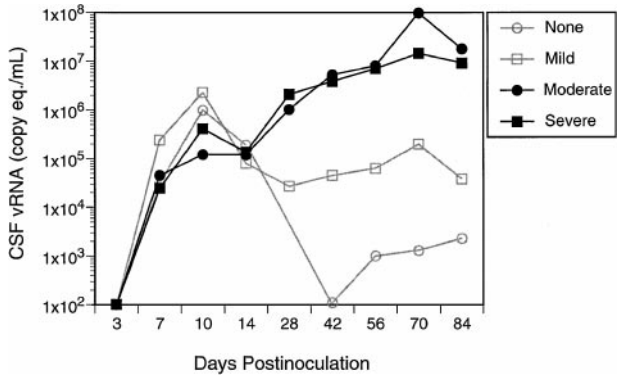

Figure 2 SIV RNA levels in CSF sampled from infected macaques every 2 weeks throughout infection, as determined by real-time RT-PCR. Lines represent mean values for macaques that developed no ($n = 1$), mild ($n = 2$), moderate ($n = 5$), or severe ($n = 4$) encephalitis.

Early virus replication in the brain: A latent reservoir

Viral RNA was quantitated by real-time RT-PCR in several anatomical sites in the brain (basal ganglia, frontal cortex, thalamus, and cerebellum) of each of the 12 infected macaques that were euthanized at 84 days p.i. Macaques with severe or moderate encephalitis had very high levels of RNA in all parts of the brain, ranging from 10^6 to $10^{7.5}$ copy equivalents per microgram brain RNA (Figure 3). Macaques with mild encephalitis had lower levels of viral RNA in the brain, ranging from 10^2 to 10^4 copy equivalents per microgram brain RNA. There was no detectable viral RNA in any site in the brain of the single macaque that did not have encephalitis (Zink *et al*, 1999). Thus, virus replication in the brain correlated with the severity of encephalitis, suggesting that SIV replication initiates the cell- and immune-mediated events that lead to CNS disease.

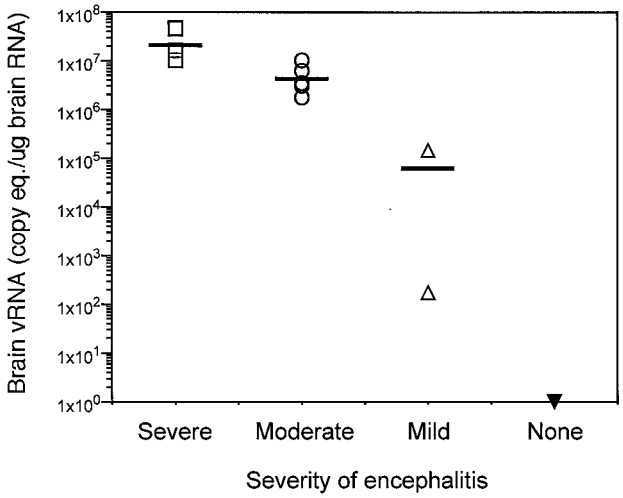

Figure 3 SIV RNA levels in brain homogenates of infected macaques euthanized at 84 days p.i. Bars represent median RNA levels.

To quantitate the extent to which virus actively replicates in the brain during acute and asymptomatic infection, six macaques were euthanized at each of 10 (acute infection), 21, and 56 (early and late asymptomatic infection, respectively) days p.i. and brain viral RNA was quantitated by real-time RT-PCR. There was significant virus replication in the brains of macaques euthanized at 10 days p.i., with viral RNA levels in the range of 50 to 2000 copy equivalents per microgram brain RNA (level of detection was 30 copy equivalents per microgram brain RNA; Figure 4) (Clements *et al*, 2002). In contrast, six of six macaques euthanized at 21 p.i. had no detectable RNA in the brain, indicating that virus replication had been suppressed or infected cells eliminated during the brief time period between 10 and 21 days p.i. Further, four of six macaques euthanized at 56 days p.i. also had no detectable virus in the brain. Viral RNA was detected in only two animals euthanized at 56 days p.i.; one macaque (369) had approximately 5×10^2 copy equivalents per microgram brain RNA in thalamus only, and the other (D7A) had an average of 5×10^4 equivalents per microgram brain RNA in all sites of brain. Taken together, these data suggest that virus was undergoing recrudescence in the brains of these two macaques. Remarkably, in this model, virus reactivates or reenters the brain at approximately 56 days p.i., leading to fulminant disease in only 28 days. The rapidity at which profound neurological changes occur in these animals demonstrates how quickly the protective mechanisms of the brain can be overwhelmed by the combination of marked replication of a neurovirulent virus and profound immunosuppression.

To determine whether virus was completely cleared from the brain or remained latent in the brains of macaques during asymptomatic infection, viral DNA was quantitated by real-time PCR in the same anatomical sites in the brain of the 18 macaques euthanized during acute and asymptomatic infection. There was substantial viral DNA in the brains of macaques euthanized at 10 days p.i. (Figure 4; 150 to 1500 copy equivalents per microgram brain DNA), and the same levels of viral DNA were detected in the brains of macaques euthanized at 21 days and in four of six macaques euthanized at 56 days p.i. (Clements *et al*, 2002). These data indicate that SIV was not cleared from the brains of infected macaques but remained latent during asymptomatic infection. Further, it suggests that the early infection of the CNS in HIV-infected individuals results in HIV latency in the brain.

This unique SIV model has provided the first demonstration that early and widespread SIV replication occurs in the CNS and is suppressed but not eliminated by immune responses in the brain. Stable levels of viral DNA in the brain suggest that there is a stable reservoir of virus in the CNS that can

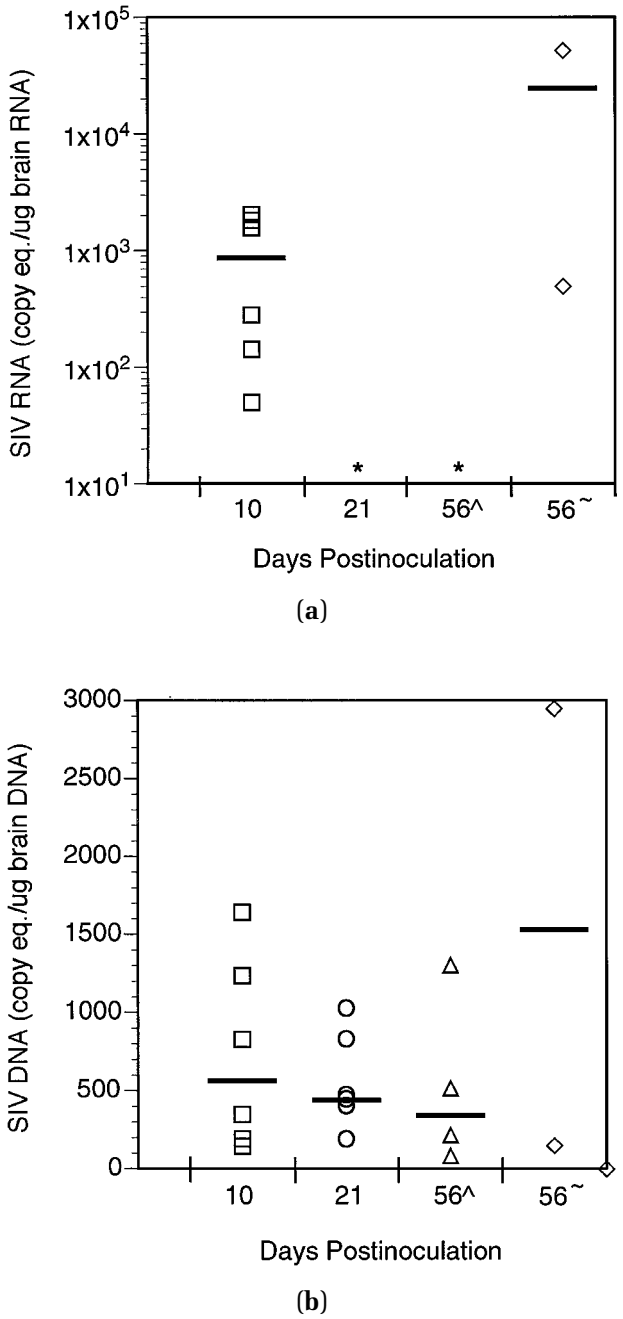

Figure 4 (**a**) SIV RNA levels in brain homogenates of infected macaques euthanized at 10, 21, and 56 days p.i. ^ represents the group of four macaques euthanized at 56 days p.i., in which no viral RNA was detected in any of the four brain sites tested. ~ represents the group of two macaques euthanized at 56 days p.i., in which viral RNA was detected in the brain. Asterisks indicate time periods at which no viral RNA was detected. Bars represent medians. (**b**) SIV DNA levels in brain homogenates of infected macaques euthanized at 10, 21, and 56 days p.i. There was no statistically significant decline in viral DNA in six of six and four of six macaques at 21 and 56 days p.i., respectively, despite the significant decline in vRNA at these time points. ^ represents the group of four macaques euthanized at 56 days p.i., in which no viral RNA was detected in any of the four brain sites tested. ~ represents the group of two macaques euthanized at 56 days p.i., in which viral RNA was detected in the brain. Bars represent medians.

reactivate and potentially reenter the peripheral circulation. Further, because the CNS is a site that limits entry of many of the antiretroviral drugs, virus replication may continue and virus may evolve independently in the CNS. This model enables us to examine which virus is present in different cellular compartments as well as to examine the evolution of virus genotypes *in vivo*.

Selective replication of neurovirulent genotypes in the brain

This unique SIV model can be used to examine how different strains of SIV establish infection in the periphery and spread to the CNS. Virus replication in the CNS can be detected as early as 7 days p.i., and examination of the virus genotypes integrated in the cellular DNA as well as replicating (viral RNA) provides a picture of the viruses that enter the CNS in trafficking lymphocytes and monocytes as well as those that are established in the brain. These studies are done longitudinally in this model so that a dynamic understanding of virus infection, spread, and reactivation can be obtained.

In this model, macaques were inoculated with a viral swarm. This enabled us to track the sites of initial virus replication and spread of viral genotypes into the brain during acute infection and to determine whether replication of specific viral genotypes in the brain is associated with the development of SIV encephalitis. Previous studies had demonstrated that genotypes of SIV/17E-Fr and SIV/DeltaB670 in blood or tissues could be differentiated by their unique V1 *env* sequences and thus this region was used as a signature for SIV/17E-Fr and for the specific strains of SIV/DeltaB670 (Zink *et al*, 1997, 1999). To evaluate the cell tropism of the viral genotypes present in the virus inoculum, we first inoculated cultured peripheral blood lymphocytes, blood-derived macrophages, and microglia isolated from uninfected brain with SIV/17E-Fr and SIV/DeltaB670. SIV/17E-Fr replicated in macrophages, lymphocytes, and microglia (Babas *et al*, 2001b). We identified four genotypes of SIV/DeltaB670 that replicated in macrophages and microglial cells and were thus deemed macrophage-tropic. An additional seven genotypes were identified that replicated in only lymphocytes were lymphocyte-tropic (Table 1) (Babas *et al*, 2001a).

Viral genotypes present in cellular DNA and RNA were indentified in all macaques euthanized during acute, asymptomatic, and terminal infections. Over 800 clones from each brain sample were screened to accurately quantitate the percentages of each genotype present. The data clearly demonstrated that SIV encephalitis is associated with the selective replication of macrophage-tropic, neurovirulent viruses. There were significantly fewer viral genotypes in the brain and in microglia than in the peripheral

Table 1 Tropism of SIV/DeltaB670 genotypes

Genotypes[a] *that replicate in*		
Lymphocytes only	*Lymphocytes and macrophages*	*Lymphocytes, macrophages, and microglial cells*
SIV/DeltaB670 Cl-4	SIV/17E-Fr	SIV/17E-Fr
SIV/DeltaB670 Cl-6	SIV/DeltaB670 Cl-2	SIV/DeltaB670 Cl-2
SIV/DeltaB670 Cl-7	SIV/DeltaB670 Cl-3	SIV/DeltaB670 Cl-3
SIV/DeltaB670 Cl-8	SIV/DeltaB670 Cl-12	SIV/DeltaB670 Cl-12
SIV/DeltaB670 Cl-14		SIV/DeltaB670 Cl-13
SIV/DeltaB670 Cl-17		
SIV/DeltaB670 Cl-22		

[a]Genotypes were based on V1 sequence analysis.

blood mononuclear cells (PBMCs) of macaques euthanized at 84 days p.i. ($P = .004$; Figure 5) (Babas *et al*, 2001a). Two macrophage-tropic genotypes, SIV/17E-Fr and SIV/DeltaB670 Cl-2, accounted for 95% of all viral genotypes detected in RNA from brain homogenates and 96% of all genotypes detected in RNA from microglia isolated from the brain. Further, each of these two genotypes was the sole genotype replicating in the brain of a macaque with encephalitis, confirming the neurovirulent phenotype of these two strains.

In contrast to the limited genotypes replicating in the brain during terminal infection, numerous macrophage- and lymphocyte-tropic genotypes were identified in brain RNA at acute infection (10 days p.i.). This suggested that there was significant trafficking of viruses from the periphery (where numerous viral genotypes were present) into the brain at this time. At 21 and 56 days p.i., although there was still a wide variety of genotypes present in PBMCs, the two neurovirulent viruses SIV/17E-Fr and SIV/DeltaB670 Cl-2 gradually increased in frequency in the brain.

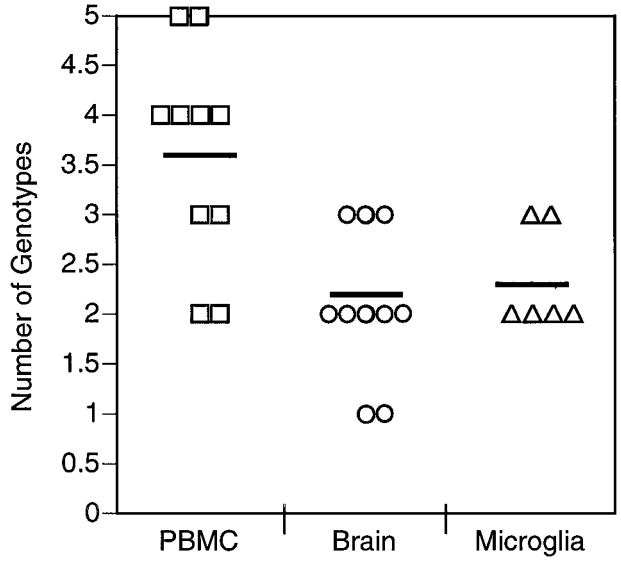

Figure 5 Number of different viral RNA genotypes detected in the PBMC, brain, and microglia of SIV-infected macaques euthanized at 84 days p.i. There were significantly fewer genotypes identified in brain and microglia than in PBMCs ($P = .004$).

The renewed virus replication that occurred in the brain of macaque D7A at 56 days p.i. provided the opportunity to determine whether the virus had reactivated from preexisting viral DNA or entered the brain from the periphery during terminal infection. SIV/17E-Fr was the major viral genotype in brain DNA and RNA, representing 94%–91% of genotypes in DNA and RNA, respectively (Clements *et al*, 2002). In addition, SIV/17E-Fr represented 93% of the genotypes present in RNA from microglial cells isolated from the brain. In contrast, SIV/17E-Fr was not detected in this macaque's PBMCs at this time. This suggests that viral DNA that is present but not actively replicating in the brain during asymptomatic infection can reactivate, resulting in virus replication, spread throughout the brain, and the development of SIV encephalitis. It also suggests that virus reactivation occurs primarily in microglial cells. Further, that SIV/17E-Fr was present in the PBMCs of most macaques at 84 days p.i. suggests that reactivated virus in the brain reenters the periphery.

MCP-1: A mechanism for inflammation and increased viral load in the CNS

A prominent histological feature of SIV encephalitis is the presence of numerous macrophages and multinucleated giant cells of macrophage origin in perivascular cuffs and throughout the brain parenchyma. These cells likely enter the CNS in response to a gradient of macrophage-attractant chemokines, with higher chemokine concentrations in the brain than in the periphery. Because chemokine expression precedes the influx of cells, we hypothesized that detection of elevated levels of macrophage-attractant chemokines in the CNS might provide a surrogate marker to predict individuals that will develop encephalitis. We therefore quantitated the comparative expression of a major macrophage attractant chemokine, macrophage chemoattractant protein-1 (MCP-1) in the CSF and plasma to determine whether a gradient of expression exists between the brain and the periphery. MCP-1 levels were expressed as the ratio of MCP-1 in CSF to plasma.

During acute infection, MCP-1 expression increased in both CSF and plasma, peaking at 10 days p.i. before declining (Figure 6) (Zink *et al*, 2001). By 28 days p.i., the CSF:plasma MCP-1 ratio had again increased in macaques that were destined to develop moderate or severe encephalitis ($n = 9$). In contrast, macaques that developed no or mild encephalitis ($n = 3$) did not demonstrate an increase in the CSF:plasma MCP-1 ratio above uninfected controls. MCP-1 ratios were significantly increased

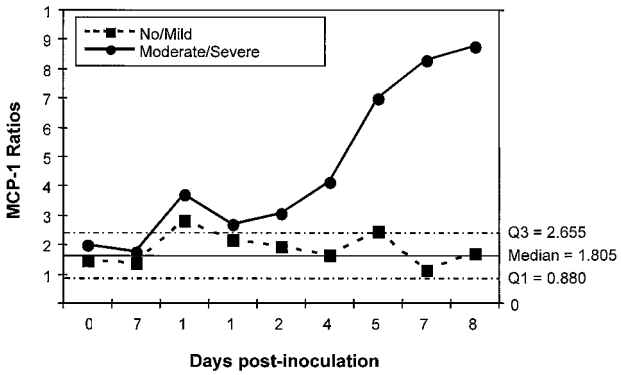

Figure 6 CSF:plasma MCP-1 ratios in infected macaques sampled every 2 weeks throughout infection. Solid lines represent macaques with moderate or severe encephalitis terminally. Dashed lines represent macaques with no or mild encephalitis terminally.

several weeks prior to the development of encephalitis. However, there was significant activation of astrocytes, a major producer of MCP-1, as early as 21 days p.i. This study thus demonstrated that early and sustained expression of MCP-1 in the brain preceded and predicted the development of SIV encephalitis. Quantitation of MCP-1 expression in the CSF and plasma should be investigated as a possible predictor of individuals at risk for developing HIV encephalitis.

Because all SIVs examined to date use CCR5 as coreceptor, chemokines that bind to CCR5 could potentially inhibit virus binding and entry to cells (Edinger *et al*, 1997b, 1998). We therefore measured the levels of the CCR5 ligands MIP-1alpha, MIP-1beta, and RANTES in CSF throughout infection. We found only moderate levels of these chemokines in the CSF, with no change in levels at any time during infection. These results suggest that differences in the level of SIV replication in the CNS is not a result of variations in the expression of these chemokines.

Conclusions

This novel SIV model has proven to be a valuable tool in unraveling the complex interactions between virus and host that result in HIV CNS disease. Using this model, we have demonstrated that CSF viral load correlates with presence and severity of SIV encephalitis and that more severe CNS lesions are associated with higher virus replication in the brain. We have further demonstrated that although virus replication is quickly suppressed after acute infection, viral DNA remains in the brain at a constant level until late infection when virus recrudesces, leading to inflammatory changes and resulting in encephalitis. Our studies have also shown that although the brain is exposed to many different viral strains during acute infection, only a limited selection of macrophage-tropic viruses replicate in macaques with encephalitis. This SIV model provides a system to examine the efficacy of anti-inflammatory and antiretroviral treatments initiated at various times after infection in ameliorating the effects of HIV in the CNS.

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