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Innate immune responses and control of acute simian immunodeficiency virus replication in the central nervous system

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Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) can invade the central nervous system (CNS) during acute infection but virus replication is apparently controlled because clinical and pathological manifestations of CNS disease in HIV/SIV-infected individuals usually present later in infection, coincident with immunosuppression and acquired immunodeficiency syndrome (AIDS). Using an established SIV/macaque model of HIV dementia, the authors recently demonstrated that acute virus replication is down-regulated (to undetectable viral RNA levels) in the brain, but not the periphery, as early as 21 days post inoculation (p.i.). Viral DNA levels in the brain remain constant, suggesting that infected cells persist in the CNS and that replication is inhibited largely at a transcriptional level. *In vitro*, active replication of HIV in macrophages can be inhibited by treatment with interferon (IFN)β via a mechanism involving induction of a dominant-negative form of the transcription factor C/EBP (CCAAT/enhancer-binding protein)β. Because macrophages are the primary cell types infected with HIV/SIV in the CNS and HIV replication in macrophages requires C/EBP sites within the viral long terminal repeat (LTR), the authors considered the possibility that suppression of C/EBP-dependent transcription contributes to the mechanism by which acute HIV/SIV replication is inhibited in the CNS. Here, the authors report that IFNβ can also inhibit ongoing SIV replication in macaque macrophages *in vitro*. Further, the authors demonstrate that IFNβ levels in the brain increase between 7 and 21 days p.i. in parallel with increased expression of the dominant-negative isoform of C/EBPβ. These results suggest that innate immune responses involving IFNβ may contribute to the mechanism(s) controlling acute SIV replication in the CNS. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 15–20.

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Introduction

Human immunodeficiency virus (HIV) can infect the brain early after infection (Davis *et al*, 1992); however, HIV-associated dementia does not typically present clinically until the development of acquired immunodeficiency syndrome (AIDS), suggesting that mechanisms exist in the central nervous system (CNS) that control HIV replication and associated virus-induced pathology during the acute and asymptomatic stages of infection. Simian immunodeficiency virus (SIV) infection of macaques provides an excellent model to investigate the cellular mechanisms that control acute virus replication in the CNS and that lead to neurological disease in HIV-infected people. SIV causes clinical and pathological manifestations similar to HIV; SIV-infected macaques develop diseases that include AIDS and associated neuropathology (Gold *et al*, 1998; Murray *et al*, 1992). The neuropathological changes induced by SIV are

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quite similar to those of HIV-infected individuals; macaques develop multifocal and perivascular aggregates of brain macrophages and multinucleated giant cells serve as the major host cells for productive replication of virus in the CNS (Hurtrel *et al*, 1991; Lackner *et al*, 1991; Sharer *et al*, 1988).

Our laboratory has developed an accelerated, consistent model of SIV CNS disease by coinoculation of a neurovirulent molecularly cloned virus, SIV/17EFr, and a virus swarm, SIV/DeltaB670 (Flaherty *et al*, 1997; Mankowski *et al*, 1997; Zink *et al*, 1997). In this model, 11 of 12 macaques developed SIV encephalitis by 3 months post inoculation (p.i.) and all macaques had AIDS. This model exhibits the classical stages of HIV/SIV disease: acute virus replication, followed by an asymptomatic stage when plasma viral load declines, and subsequent resurgence of virus replication in brain concomitant with the development of AIDS. During the final stage, the infected macaques develop inflammation in the CNS that correlates with high virus load in brain and elevated levels of monocyte chemoattractant protein-1 in cerebrospinal fluid, also characteristics of HIV-associated neuropathology (Brew *et al*, 1997; Ellis *et al*, 1997; McArthur *et al*, 1997; Zink *et al*, 1999, 2001).

In our model, we have examined early events in the CNS that accompany acute and asymptomatic infection (Clements *et al*, 2002). Our results indicate that acute virus replication in the CNS (10 days p.i.) occurs simultaneously with activation of macrophages and microglia. Interestingly, in all macaques examined, dramatic down-regulation of virus replication (i.e., viral RNA) and macrophage activation markers occurred between 10 and 21 days p.i. in the CNS, despite continued virus replication in the periphery during this time period. In contrast, viral DNA levels were unchanged between 10 and 21 days p.i., suggesting that virus-infected cells were not eliminated in the brain; rather, virus gene expression was down-regulated. These findings suggest that mechanisms exist in the CNS that control SIV replication and associated macrophage activation early after infection.

Active replication of HIV in primary macrophages *in vitro* can be inhibited by treatment with interferon (IFN)β (Honda *et al*, 1998; Kornbluth *et al*, 1990, and references therein). IFNβ is a type 1 IFN and an integral component of innate immunity, particularly with regard to antiviral immune responses (Biron, 2001). Elevated levels of IFNβ in plasma have been described during infection with HIV and SIV (Abel *et al*, 2002; Giavedoni *et al*, 2000; Minagawa *et al*, 1989), but no information currently exists regarding IFNβ expression in the CNS of infected individuals. Because macrophages and microglia are the predominant cells infected by HIV and SIV in the brain, the observations that IFNβ can inhibit ongoing HIV replication in primary macrophages suggested that induction of IFNβ in the brain may participate in the mechanism(s) mediating down-regulation of acute virus replication in the CNS *in vivo*.

Active replication of HIV in primary macrophages and differentiated promonocytic cell lines *in vitro* (but not lymphocytes/cell lines) requires C/EBP- (CCAAT/enhancer-binding protein) transcription factors as well as one of the C/EBP binding sites in the viral long terminal repeat (LTR), and recent studies have suggested the involvement of C/EBP proteins as well as C/EBP binding sites in the viral LTR in HIV-associated neuropathogenesis (Henderson and Calame, 1997; Hogan *et al*, 2002, and references therein). Thus, it is intriguing that the mechanism by which IFNβ inhibits active HIV replication in macrophages has been shown to involve the induction of a dominant-negative form of the transcription factor C/EBPβ (Honda *et al*, 1998; Weiden *et al*, 2000). Dominant-negative C/EBPβ, a truncated form of the wild-type protein likely produced by a ribosome scanning mechanism, has been shown not only to suppress C/EBPβ-mediated HIV-1 transcription, but also to inhibit nuclear factor (NF)-κB–mediated transcriptional activation (Descombes and Schibler, 1991; Ossipow *et al*, 1993; Prosch *et al*, 2001). Because truncated C/EBPβ can bind DNA (e.g., the albumin D promoter) with higher affinity, a modest shift in the ratio of wild-type/truncated C/EBPβ favoring expression of the truncated form is sufficient to mediate profound transcriptional suppression. Moreover, although the truncated C/EBPβ lacks a functional activation domain, it retains both DNA-binding and dimerization domains such that homodimeric complexes of truncated C/EBPβ as well as heterodimeric complexes of wild-type/truncated C/EBPβ heterodimers inhibit transcription (Ossipow *et al*, 1993).

In this study, we examined the effect of IFNβ on SIV replication and demonstrate that treatment of SIV-infected macaque macrophages with IFNβ inhibits active virus replication. Further, because macrophages are the predominant infected cells in the brain and IFNβ induces an isoform of the transcription factor C/EBPβ that inhibits HIV replication in macrophages, we examined the expression of IFNβ and C/EBPβ during acute SIV infection of the CNS at 7, 10, and 21 days p.i. These time points correspond to active viral replication in the CNS (7 and 10 days p.i.) and down-regulated viral replication (21 days p.i., Clements *et al*, 2002). Our results indicate that IFNβ levels in the brain increase between 7 and 21 days p.i., in parallel with increased expression of the dominant-negative isoform of C/EBPβ. Our results suggest that innate immune responses involving IFNβ may contribute significantly to the mechanism(s) controlling acute SIV replication in the CNS.

Results

IFNβ inhibits SIV replication in primary macaque macrophages

IFNβ has been shown to inhibit HIV replication in macrophages *in vitro*, by a mechanism involving

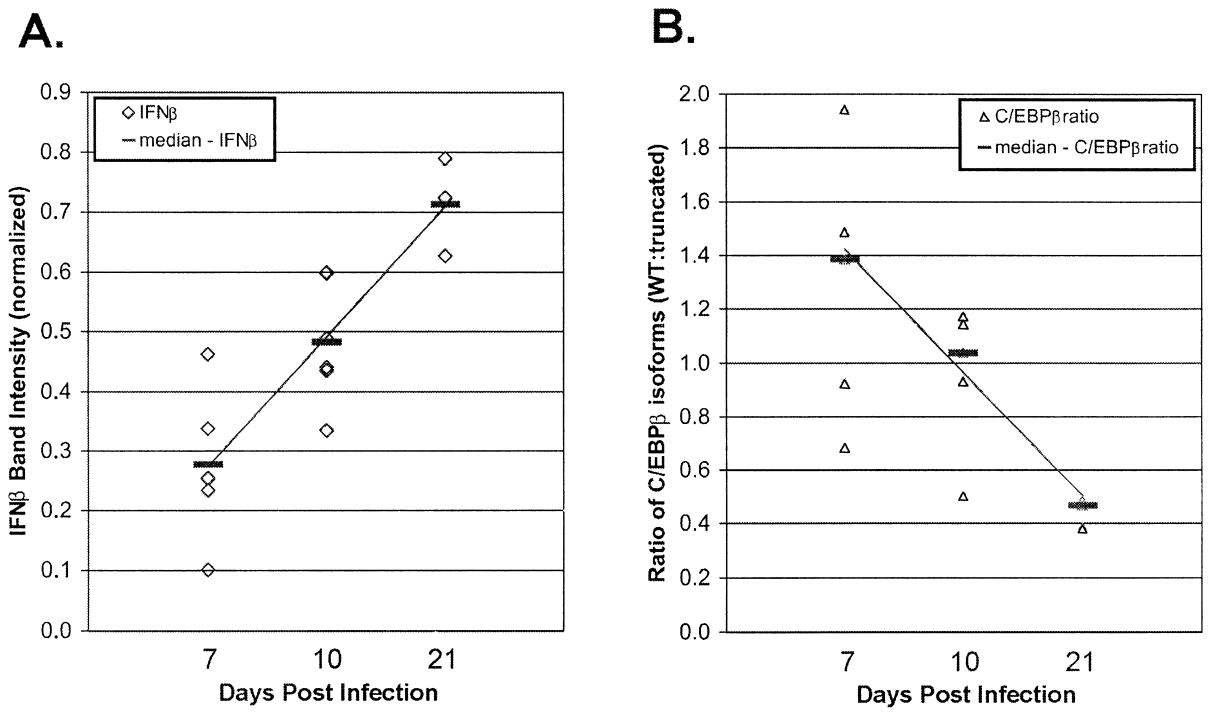

Figure 1 Western blot analysis of IFNβ and C/EBPβ expression in the CNS. Tissue homogenates were prepared from basal ganglia of SIV-infected macaques euthanized at the indicated times post infection. Quantitative Western blot analysis was performed as described in Materials and Methods. **A**, IFNβ expression in tissue samples was normalized to 0.5 μg human recombinant IFNβ present on each gel. IFNβ expression (left *y*-axis) is statistically different at each day post infection as assessed by the two-sample *t* test ($P = .026$ [7 versus 10 day]; $P = .002$ [7 versus 10 day]; $P = .015$ [10 versus 21 day]). **B**, Wild-type (WT) C/EBPβ (37 kDa) expression was divided by expression of the dominant-negative (truncated) C/EBPβ (16 kDa) to generate a ratio (WT:truncated) for each animal. C/EBPβ expression (right *y*-axis) at 21 days p.i. is statistically different than C/EBPβ expression at 7 and 10 days as assessed by the two-sample *t* test ($P = .02$ [7 versus 21 days)]; $P = .015$ [10 versus 21 days]).

increased expression of a dominant-negative form of the transcription factor C/EBPβ. In addition, the abilities of proinflammatory mediators, mitogens, and pathogens, such as tumor necrosis factor (TNF)-α, lipopolysaccharide (LPS), and *Mycobacterium tuberculosis*, to inhibit active replication of HIV in macrophages are also dependent on the production of IFNβ and increased expression of the dominant-negative C/EBPβ (Honda *et al*, 1998, and references therein). To evaluate the ability of IFNβ to inhibit SIV replication in macrophages, peripheral blood-derived macaque macrophages were infected for 3 days with SIV/17EFr and then treated or not for 3 days with recombinant human IFNβ (1 U/ml). Treatment with IFNβ reduced reverse transcriptase (RT) activity in the supernatants of SIV/17EFr-infected macrophages by 80% (data not shown). These results clearly demonstrate the ability of IFNβ to inhibit ongoing replication of SIV in macrophages.

IFNβexpression increases in the brains of SIV-infected macaques between 7 and 21 days p.i.

We next examined the expression of IFNβ in the brains of SIV-infected macaques that were euthanized at 7, 10, or 21 days p.i. Brain homogenates were prepared from the same macaques sacrificed at 10 and 21 days p.i. (Clements *et al*, 2002), as well as six additional macaques infected and sacrificed at 7 days p.i. Western blot analysis of each gel and quantitation of the intensities of the IFNβ-specific bands (Figure 1A) demonstrate that IFNβ levels increase steadily from 7 to 21 days p.i. Of note, the expression of IFNβ in individual macaques at each time point are remarkably similar, suggesting that the induction of IFNβ in the brain may be a consistent and coordinated host response to SIV infection of the CNS.

Increased expression of truncated C/EBPβ in the brains of SIV-infected macaques occurs between 7 and 21 days p.i.

To determine whether an association exists between the expression of IFNβ and the truncated form of C/EBPβ in the CNS during acute infection, we next examined expression of C/EBPβ in brain homogenates obtained from SIV-infected macaques that were euthanized at 7, 10, or 21 days p.i. (Clements *et al*, 2002). Wild-type C/EBPβ migrates to ~37 kDa in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), whereas the truncated form of C/EBPβ migrates to ~16 kDa, thus enabling quantitation of both C/EBPβ isoforms. Following Western blot analysis of each gel, the intensities

of the C/EBPβ-specific bands were the quantitated and intensity of wild-type C/EBPβ present in each homogenate sample was divided by the intensity of the truncated form of C/EBPβ. The ratios of wild-type to truncated isoforms of C/EBPβ (Figure 1B) demonstrate that a shift in the ratio of wild-type to truncated C/EBPβ, in favor of increased expression of the truncated C/EBPβ isoform (and hence a lower ratio), occurs from 7 to 21 days p.i., in parallel with increasing expression of IFNβ. In fact, the ratios of wild-type to truncated C/EBPβ in individual macaques at each time point become closer with increasing expression of IFNβ, which would be expected if an association exists between the expression of IFNβ and the expression of truncated C/EBPβ *in vivo*.

Discussion

In our SIV model, all infected macaques down-regulate virus replication between 10 and 21 days p.i. in the CNS, as evidenced by a transition to undetectable levels of viral RNA (Clements *et al*, 2002). During this period of time, it is reasonable to attribute detectable levels of viral RNA to productively infected resident CNS cells as well as productively infected trafficking monocytes. However, the fact that no decrease in viral DNA levels is observed suggests that infected cells are not eliminated and that at least one component of the innate immune response to infection of the CNS inhibits ongoing viral transcription. In addition to transcriptional repression, other innate inhibitory mechanisms likely function in parallel to suppress viral spread and entry of infected immune cells across the blood-brain barrier (BBB), in order to secure efficient control of acute virus replication in the CNS.

IFNβ has been shown to down-regulate several stages of virus replication: uptake of virus particles, reverse transcription, integration, transcription, translation of viral proteins, packaging and release of viral particles, and infectivity of progeny virions (Korth *et al*, 1998, and references therein). IFNβ also induces up-regulation of chemokines (including RANTES) in macrophages and microglia and down-regulation of CCR5, potentially decreasing effective HIV/SIV infection or spread (Cremer *et al*, 2000). Further, in addition to antiviral activities, IFNβ also suppresses production of proinflammatory cytokines (in favor of anti-inflammatory products such as interleukin [IL]-1 receptor antagonist) and inhibits leukocyte migration across the BBB by downregulating intercellular adhesion molecule (ICAM)-1 expression in endothelial cells and matrix metalloproteinase-9 activity (Floris *et al*, 2002; Kim *et al*, 2002; Ma *et al*, 2001, and references therein). In this regard, it is not surprising that the temporal increase in expression of IFNβ in the CNS observed in our model correlates not only with repressed virus transcription but also with down-regulation of activation markers such as CD68 on macrophage/microglia and ICAM-1 on endothelial cells (Clements *et al*, 2002; unpublished observations).

In our view, the consistent and rapid control of acute virus replication by innate immune responses in the CNS may be crucial to defer the onset of CNS disease because although there are alterations in the BBB as well as expression of proinflammatory cytokines and viral proteins (all associated with late stage infection) early after infection, there is no overt sign of neuropathology. Perhaps intrinsic to the ability of innate immune responses in the CNS to inhibit acute virus replication is the lack of a well-developed adaptive immune response (in our model, TIA-1–positive cells appear at 21 days p.i., yet there is no evidence of virus-specific cytolytic activity because levels of viral DNA in the brain remain unchanged for at least 56 days p.i., Clements *et al*, 2002). Intriguingly, a similar scenario has been described with regard to IFNβ- and C/EBPβ-mediated repression of HIV-1 replication in the lung, in which repression of virus transcription becomes ineffective in the presence of adaptive immune cells (Honda *et al*, 1998; Hoshino *et al*, 2002). In these studies, activated $CD4^+$ or $CD8^+$ T lymphocytes and associated cytokines down-regulate expression of truncated C/EBPβ and activate NF-κB in alveolar macrophages, culminating in transcriptional activation of HIV. It will be important to examine whether this occurs in the CNS during the later stages of infection when there is a resurgence of virus replication in the brain that is accompanied by the influx of cytotoxic lymphocytes and the development of lesions.

Given the complexity of innate immune responses, it seems unlikely that IFNβ is the only important contributor to suppression of acute SIV transcription in the CNS. Defensins, recently implicated in non-cytolytic suppression of virus replication by $CD8^+$ T cells (Zhang *et al*, 2002, and references therein), and transforming growth factor (TGF)-β (Coyle-Rink *et al*, 2002) have been shown to inhibit transcription of HIV-1 and, thus, may also contribute to the efficacy with which the CNS controls acute virus replication. We are currently examining these and other possibilities in our SIV model. Identification of mechanisms that control virus replication during acute infection will provide the basis for experiments examining dysregulation of virus replication during late-stage CNS disease and perhaps elucidate a logical approach for therapeutic intervention.

Materials and methods

Cells and virus stocks

Primary rhesus macaque macrophages were derived from peripheral blood obtained from adult macaques

and cultured as previously described (Flaherty *et al*, 1997). SIV/17EFr, a neurovirulent macrophage-tropic clone, has been characterized (Flaherty *et al*, 1997). Virus stocks of SIV/17EFr were prepared by transfection of infectious DNA of the clone into CEM × 174 cells as previously described (Flaherty *et al*, 1997).

Reagents
Recombinant human IFNβ and antiserum against huIFNβ was obtained from R&D Systems (Minneapolis, MN). Antiserum against C/EBPβ (recognizes wild-type and truncated isoforms) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse transcriptase assay
The assay for RT activity has been described previously (Flaherty *et al*, 1997).

SIV infection of macaques
Macaques were inoculated intravenously with 10,000 AID_{50} of SIV/17EFr and 50 AID_{50} of SIV/DeltaB670 as previously described; this inoculation protocol has historically resulted in SIV encephalitis in over 90% of macaques by 84 days p.i. (Zink *et al*, 1999, 2001).

Western blots
Brain homogenates were prepared from basal ganglia and protein concentrations were determined as previously described (Barber *et al*, 2002). Equal amounts of protein were separated by SDS-PAGE and analyzed for expression of IFNβ and C/EBPβ using standard Western blot technology in conjunction with the antisera described above. Band intensities were quantitated using the Kodak Image Station as described previously (Barber *et al*, 2002).

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