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# Decreased neurotropism of *nef* long terminal repeat (*nef*/LTR)-deleted simian immunodeficiency virus

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**Simian immunodeficiency virus (SIV) infection of macaques results in neurological abnormalities similar to those of human immunodeficiency virus (HIV)-associated dementia in humans and is a valuable system for the identification of viral neurotropic and neurovirulence factors. The authors recently established an SIV-macaque model where macaques can be infected with wild-type or *nef*/LTR-deleted SIVmac239 via administration of purified proviral DNA. In this study, the ability of wild-type and *nef*/LTR-deleted SIV infections to enter the cerebral spinal fluid (CSF) and brain was analyzed. *In situ* polymerase chain reaction (PCR) readily detected SIV *gag* DNA–positive cells in the midfrontal gyrus and basal ganglia of the wild-type SIV-infected macaques, but not in *nef*/LTR-deleted SIV-infected or SIV-uninfected macaques. PCR on extracted DNA confirmed the *in situ* results, with multiple brain regions of the wild-type SIV-infected macaques positive for both *gag* and wild-type *nef*, whereas in the *nef*/LTR-deleted SIV-infected macaques, *nef*/LTR and *gag* DNA were undetectable. Further, macaques infected with *nef*/LTR-deleted SIV, which later became superinfected with wild-type SIV, also remained negative for SIV DNA in the brain by both *in situ* and extracted DNA techniques, despite having high levels of SIV RNA both in the CSF and plasma. This study provides evidence of the inability of *nef*/LTR-deleted SIV to initiate central nervous system (CNS) infection and suggests that, in the brain regions examined, *nef*/LTR-deleted viruses have either diminished neurotropism or insufficient systemic viral replication for entry into the CNS.** *Journal of NeuroVirology* (2003) **9,** 442–451.

**Keywords:** HIV-associated dementia; macaque; *nef*; neuropathogenesis; neurotropism; simian immunodeficiency virus

## Introduction

Human immunodeficiency virus (HIV)-associated dementia (HIVD) is the most common cause of dementia in people under the age of 40 (Sacktor *et al*, 2001). The development of neurological disease in an HIV-infected individual appears to depend on the net result of both viral virulence and host factors. Important viral factors include cell tropism and genetic sequences that determine neurovirulence, and host factors include cellular expression of viral coreceptors and the dysregulation of the immune response (von Herrath *et al*, 1995; Zink *et al*, 1998).

Simian immunodeficiency virus (SIV) infection results in neurological abnormalities in macaques that are clinically and pathologically similar to HIVD in humans (Adamson *et al*, 1996; Demuth *et al*, 2000; Zink *et al*, 1997, 1998). The SIV-macaque model allows identification of viral virulence factors because macaques can be inoculated with well-characterized

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virus strains or molecular clones of SIV to identify specific viral genes that are important in the development of neurological disease. We have recently shown that SIV infection can be initiated by inoculation of wild-type or attenuated purified plasmid SIV DNA by either intramuscular (IM) or gene gun inoculation (Kent *et al*, 2001). This facilitates molecular analysis of SIV neurotropism.

A few percent of HIV-1–infected individuals remain free from acquired immunodeficiency syndrome (AIDS)-defining illnesses for extended periods in the absence of therapy. These individuals, known as long-term nonprogressors (LTNPs), maintain CD4+ lymphocyte counts greater than 500 cells/$\mu$l and low plasma HIV-1 RNA levels without receiving therapy (Buchbinder *et al*, 1994). Several defects in the viral genome of HIV-1 strains infecting LTNPs have been reported, with the most common being deletions in the *nef* gene or deletions in the *nef* long terminal repeat (*nef*/LTR) overlap region (Iversen *et al*, 1995; Mariani *et al*, 1996; Salvi *et al*, 1998). The largest study of LTNPs infected with a defective HIV-1 strain has been the Sydney Blood Bank Cohort in Australia (Deacon *et al*, 1995; Learmont *et al*, 1999; Rhodes *et al*, 1999). This cohort consists of nine individuals who became infected with a *nef*/LTR-deleted HIV-1 strain after being transfused with blood products from a common donor. Previous studies suggest sequences in *nef* and *env* genes are important determinants of neurovirulence of diverse HIV-1 strains in humans (Flaherty *et al*, 1997) and sequence changes in these genes may account for differences in tropism *in vitro* and neurovirulence *in vivo.*

To address whether the *nef*/LTR overlap region is important for viral entry and replication in the central nervous system (CNS), we studied the entry of SIV into the cerebrospinal fluid (CSF) and brain of macaques inoculated with purified DNA expressing either wild-type SIV or SIV containing deletions in the *nef*/LTR overlap region analogous to the Sydney Blood Bank Cohort deletion.

## Results

### *SIV infection of macaques*

The CNS infection of six pigtail macaques inoculated with wild-type or *nef*/LTR-deleted SIV mac239 DNA constructs was studied (Table 1). The *nef*/LTR-deleted SIV constructs ($SIV_{sbbc\Delta3}$ or $SIV_{sbbc\Delta3\Delta5}$) were constructed to mimic the minimal common *nef*/LTR deletion present in the HIV-1 strains from the Sydney Blood Bank Cohort (Figure 1). An additional macaque infected intrarectally with SIVmac251 and four SIV-uninfected animals were also studied (Kent *et al*, 1997, 1998, 2001, 2002). All SIV-inoculated animals seroconverted to SIV, and had SIV recovered by peripheral blood monocular cell (PBMC) cocultures and SIV RNA detected in plasma (Figure 2) (Kent *et al*, 2001). The SIV-inoculated animals were divided into three groups based on the virologic and immunologic outcome of their infection: (a) wild-type SIV infection that progressed to simian AIDS (M12, M13, M20); (b) *nef*/LTR-deleted SIV infection that did not progress to AIDS (M14, M17); and (c) animals inoculated with *nef*/LTR-deleted SIV that reverted to wild-type SIV or were subsequently superinfected with wild-type SIV following challenge (M16, M18).

**Table 1** Comparison of SIV infection in the macaque groups

| *Group* | *Macaque* | *SIV inoculated* | *Plasma SIV RNA* | *CD4 T-cell count* | *SIV infection* | *Brain examination (week following SIV inoculation)* |
|---|---|---|---|---|---|---|
| SIV wild-type | M12 | $SIV_{mac239}$DNA | High | Low | }Progressed to AIDS | 53 |
| | M13 | $SIV_{mac239}$ DNA | High | Low | | 19 |
| | M20 | $SIV_{mac251}$ | High | Normal | Died during acute SIV infection | 3 |
| SIV *nef*/LTR deleted | M14 | $SIV_{sbbc\Delta3}$ DNA | Low | Normal | }Remained healthy with *nef*/LTR-deleted SIV | 98 |
| | M17 | $SIV_{sbbc\Delta3\Delta5}$ DNA | Low | Normal | | 67 |
| SIV *nef*/LTR deleted reverted to SIV wild-type | M16 | $SIV_{sbbc\Delta3}$ DNA | Low to high[a] | Normal to low | *nef*/LTR-deleted SIV, reverted to wild-type at week 12 then progressed to AIDS | 61 |
| | M18 | $SIV_{sbbc\Delta3\Delta5}$ DNA | Low to high[a] | Normal to low | Challenged with SIVmac251 at week 39 became infected and progressed to AIDS | 67 |
| SIV negative | M1 | Non pathogenic $HIV\text{-}1_{LAI}$ | Negative | Normal | }SIV uninfected, healthy | NA |
| | M4 | Non pathogenic $HIV\text{-}1_{LAI}$ | Negative | Normal | | NA |
| | M6 | Non pathogenic $HIV\text{-}1_{LAI}$ | Negative | Normal | | NA |
| | M9 | Non pathogenic $HIV\text{-}1_{LAI}$ | Negative | Normal | | NA |

NA = not applicable.
[a]Macaques M16 and M18 started with an attenuated SIV infection but the infection later progressed to a wild-type SIV infection.

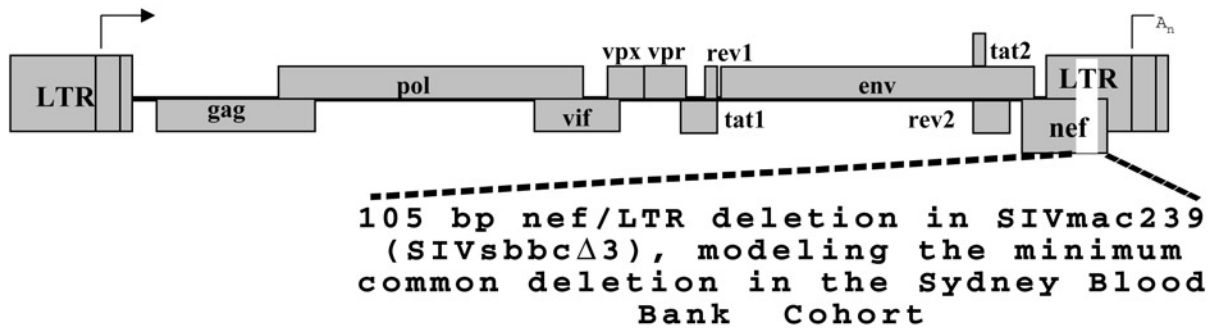


**Figure 1** An infectious molecular clone of SIVmac 239 with a 105-bp deletion in the 3′ *nef*/LTR overlap region ($SIV_{sbbc\Delta 3}$) was engineered (Kent *et al*, 2001) analogous to HIV-1 strains isolated from the Sydney Blood Bank Cohort of long-term nonprogressors with similar *nef*/LTR deletions in HIV-1 (Deacon *et al*, 1995).

*CSF and plasma SIV viral loads*

To assess the levels of SIV replication in the periphery and CSF compartments, real-time reverse transcriptase–polymerase chain reaction (RT-PCR) or bDNA assays were used to quantify SIV RNA in the CSF and plasma as previously reported (Kent *et al*, 2001) (Figure 2, Table 2). The lower detection limit of these assays is 1500 RNA copies or 3.18 log copies per milliliter. Wild-type SIV-infected macaques had high levels of SIV RNA in the CSF concomitant with high (>$10^5$ copies/ml) levels of SIV RNA in the plasma (Figure 2**C**). The macaques infected with *nef*/LTR-deleted SIV had undetectable (<1500 copies/ml) levels of SIV RNA in the CSF, concomitant with lower or undetectable levels of plasma SIV RNA, following the initial acute infection (Figure 2**A**). The two animals initially infected with *nef*/LTR-deleted SIV, but which reverted to, or became super-infected with, wild-type SIV, initially had low levels of SIV RNA in the CSF, but following reversion to wild-type SIV, had high levels of CSF RNA, following the pattern observed in the plasma (Figure 2**B**).

*Brain histopathology and presence of SIV DNA in the brain*

To examine the effects of wild-type or attenuated SIV infections on the macaque brains, the brains were removed at autopsy and examined by histopathology, for SIV *gag* and SIV *nef* DNA on extracted brain DNA samples, and for SIV *gag* DNA by *in situ* PCR. The animals were autopsied at the times noted in Table 1, either with incipient simian AIDS in the case of the macaques infected with wild-type SIV or those that reverted to wild-type SIV, or with asymptomatic SIV in the case of the two animals with persisting *nef*/LTR-deleted strain and the

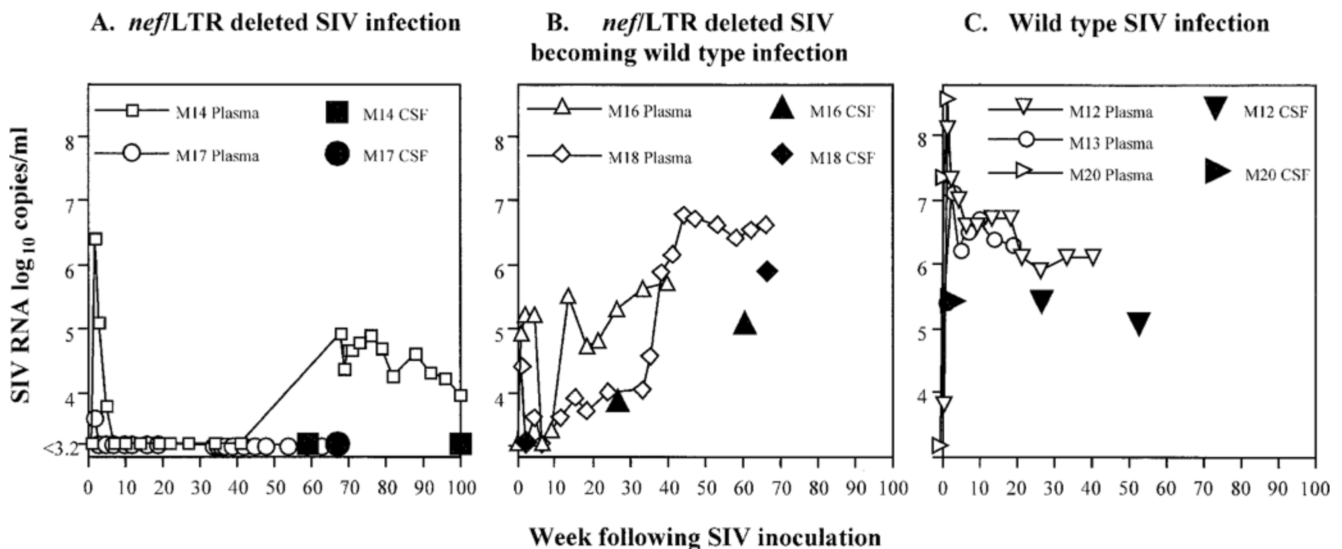


**Figure 2** Plasma and CSF SIV RNA levels. Plasma SIV RNA (*open symbols*) was followed at regular intervals following SIV inoculation of macaques by real-time RT-PCR and bDNA as previously described (Kent *et al*, 2001). CSF SIV RNA (*closed symbols*) was studied at autopsy and on some animals at an additional earlier time point by real-time RT-PCR. The animals were divided into three groups based on their outcome of infection: (**A**) attenuated SIV infection; (**B**) attenuated SIV infection that reverted to wild-type SIV and progressed to AIDS; or (**C**) wild-type SIV infection.

**Table 2** Neuropathology and PCR detection of SIV *gag* and *nef* DNA in brain regions and CSF of macaque groups

| | | | | | | | gag in situ *PCR*[e] | | *SIV RNA in CSF*[f] | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Group*[a] | *n* | *Macaque* | *Neuropathology*[b] | nef *PBMC PCR*[c] | nef *brain PCR*[d] | gag *brain PCR*[d] | *MFG* | *BG* | *Early* | *Late* |
| SIV wild-type | 3 | M12 | ++ | Wild-type *nef* | – | – | +++ | +++ | $2.4 \times 10^5$ | $1.1 \times 10^5$ |
| | | M13 | ++ | Wild-type *nef* | Wild-type *nef* | – | ++ | ++ | NA | NA |
| | | M20 | + | Wild-type *nef* | Wild-type *nef* | SIV *gag* | +++ | +++ | $2.3 \times 10^5$ | NA |
| SIV *nef*/LTR deleted | 2 | M14 | + | *nef*/LTR deleted | – | – | – – | – | Undetectable | Undetectable |
| | | M17 | – | | – | – | – | – | NA | Undetectable |
| SIV *nef*/LTR deleted reverted to SIV wild-type | 2 | M16 | + | *nef*/LTR deleted reverted to wild-type *nef* | – | – | – | – | $6.9 \times 10^3$ | $1.1 \times 10^5$ |
| | | M18 | + | | – | – | – | – | Undetectable | $7.5 \times 10^5$ |
| SIV negative | 4 | M1 | – | – | – | – | – | – | NA | NA |
| | | M4 | – | – | – | – | – | – | Undetectable | NA |
| | | M6 | – | – | – | – | – | – | NA | NA |
| | | M9 | – | – | – | – | – | – | Undetectable | NA |

[a]Macaques were infected with wild-type or *nef*/LTR-deleted SIVmac239 via administration of purified proviral DNA. Macaques were divided into 4 groups: (1) wild-type SIV infection that progressed to simian AIDS (M12, M13, M20); (2) *nef*/LTR-deleted SIV infection that did not progress to AIDS (M14, M17); (3) macaques inoculated with *nef*/LTR-deleted SIV that either reverted to wild-type SIV or were subsequently superinfected with wild-type SIV following challenge (M16, M18); and (4) SIV-seronegative macaques (M1, M4, M6, M9).
[b]Neuropathology showed inflammation with lymphocytic cuffing of meninges, increased meningeal fibrosis, and lymphocytic meningitis, the extent to which is assigned: – = no pathology; + = minimal, ++ = extensive.
[c]Presence of SIV *nef* wild-type or *nef*/LTR-deleted DNA consistently detected by nested PCR of peripheral blood mononuclear cells.
[d]Presence of SIV *nef* or *gag* DNA consistently detected by PCR in multiple brain region samples (temporal, parietal, frontal lobes, and mid-brain).
[e]Qualitation of *in situ* PCR detection of SIV *gag* DNA in brain cells in the mid-frontal gyrus and basal ganglia by assigning: – = no positive cells; + = ≤0.02 cells/mm$^2$; ++ = 0.03–0.05 cells/mm$^2$; +++ = ≥0.05 cells/mm$^2$.
[f]Quantitation of SIV *gag* RNA in the CSF by real-time RT PCR. Undetectable = the lower detection limit of these assays is 1500 RNA copies or 3.18 log copies per ml. NA = no CSF sample available.
*n* = number of macaques examined per group.

four control animals. By histopathology, the three wild-type SIV–inoculated macaques showed positive brain inflammation, with lymphocytic cuffing of meninges, increased meningeal fibrosis, and lymphocytic meningitis (Figure 3); however, there was no evidence of neuronal loss, gliosis, or multifocal glial nodules. Both animals initially infected with the *nef*/LTR-deleted SIV strain, but which reverted to wild-type infections with high levels of SIV RNA in the plasma and CSF and progressed to AIDS, also showed positive brain inflammation. In contrast, the two animals with persisting attenuated *nef*/LTR-deleted SIV infection, which had low or undetectable plasma and CSF SIV RNA levels, showed either no evidence of brain inflammation by histopathology (monkey 17), or very minimal inflammation (monkey 14) consisting of very scant lymphocytic cuffing of basal ganglia. There was no evidence of brain inflammation by histopathology in the four SIV-uninfected controls.

The presence of SIV DNA in the brain was examined by standard and *in situ* PCR. *In situ* PCR was performed on multiple 6-$\mu$m serial sections of paraffin-embedded tissue from the mid-frontal gyrus and basal ganglia of each monkey to detect SIV *gag* DNA. These serial sections showed reproducible detection of SIV *gag* DNA–positive cells, which were averaged for an overall qualitative measure for the two areas of each monkey (Table 2). Appropriate control samples were included in each run of the *in situ* PCR, including serial sections from the same tissue blocks, which were treated without Taq DNA polymerase or oligonucleotide probes, or with irrelevant primer pairs or irrelevant oligonucleotide probes. Tissue sections from an SIV-negative macaque were also included in each run. These controls, included to identify any false positives, did not detect any SIV *gag* DNA–positive cells. SIV *nef* and SIV *gag* PCR methods on brain tissue were adapted from the PCR analyses performed in PBMCs and were able to differentiate wild-type and *nef*/LTR-deleted strains (Kent *et al*, 2001, 2002). Table 2 summarizes the PCR results for the three SIV-infected groups (wild-type SIV, *nef*/LTR-deleted SIV, and *nef*/LTR-deleted SIV becoming wild-type SIV). Wild-type SIV–infected macaques were positive for wild-type *nef* in the PBMCs and for both wild-type *nef* and *gag* DNA in the extracted brain tissue, which included temporal, parietal, and frontal lobes and midbrain. *In situ* PCR also detected numerous SIV *gag* DNA–positive cells in the mid-frontal gyrus and basal ganglia (Figure 3). Although *nef*/LTR-deleted SIV–infected macaques were positive for low levels of *nef*/LTR-deleted DNA in PBMCs (Kent *et al*, 2001), neither *nef*/LTR-deleted or *gag* DNA was detected in the brain tissue and no SIV *gag* DNA was detected by *in situ* PCR. The SIV-uninfected macaque group were also negative for SIV DNA in all of the PCR procedures. In the subgroup of *nef*/LTR-deleted infected macaques that reverted to wild-type SIV, the PBMC PCR of blood samples collected over time showed reversion to wild-type SIV (Kent *et al*, 2001). However, as with the SIV *nef*/LTR-deleted macaques that did not revert to wild-type, we were unable to detect *nef* or *gag* DNA in either the extracted brain tissue PCR or the *in situ* PCR. However, we did detect a small number of SIV-positive cells that appeared to be blood monocytes in the blood vessels of the mid-frontal gyrus.

## Discussion

The requirements for the development of HIVD appear to be immunosuppression, CNS viral replication, and a neurovirulent virus. *nef* may be an important factor in all three of these requirements. In order to further clarify the role of *nef* in the development of HIVD and to give insights that cannot be gained from *in vitro* studies or human investigations, we have examined brain tissue from macaques infected with SIV molecular clones.

In this study, macaques were infected with SIV with a 3′ *nef*/LTR overlap region deletion, analogous to the Sydney Blood Bank Cohort deletion (Rhodes *et al*, 2000; Kent *et al*, 2001). *In situ* PCR, nested PCR, and real-time RT PCR were utilized to determine if SIV was present in CSF samples and postmortem brain tissue from macaques infected with wild-type and *nef*/LTR-deleted SIV. In contrast to wild-type SIV, which was found in the CSF and brain regions examined, *nef*/LTR-deleted SIV was not detected in the CSF or brain regions examined. This suggests *nef*/LTR-deleted viruses may have either diminished neurotropism or insufficient systemic viral replication and/or immunosuppression for entry into CNS.

HIV isolated from the CNS of patients with HIVD is usually macrophage-tropic, but not all individuals infected with macrophage-tropic strains of HIV develop neurological disease (Adamson *et al*, 1996; Flaherty *et al*, 1997). This suggests that not all macrophage-tropic viruses are neurovirulent and that other viral factors may play a role in the development of CNS disease (Clements *et al*, 1994). Viruses containing deletions in the *nef* gene cause infection in rhesus macaques but not progression to simian AIDS (Kestler *et al*, 1991). *In vivo* passaged neurovirulent strains of SIVmac239 have been mapped for the molecular determinants of neurovirulence. These studies demonstrated that macrophage tropism alone is not sufficient for the development of neurological disease, and that although sequences in the surface portion of the envelope gene determine macrophage tropism, additional sequences derived from the transmembrane portion of envelope and/or *nef* confer neurovirulence (Flaherty *et al*, 1997; Mankowski *et al*, 1997; Zink *et al*, 1997). Interestingly, SIV with just the *env* gene could be detected in the brains of macaques but they could not initiate disease. However, we did not detect *nef*-deleted SIV DNA in brain tissue

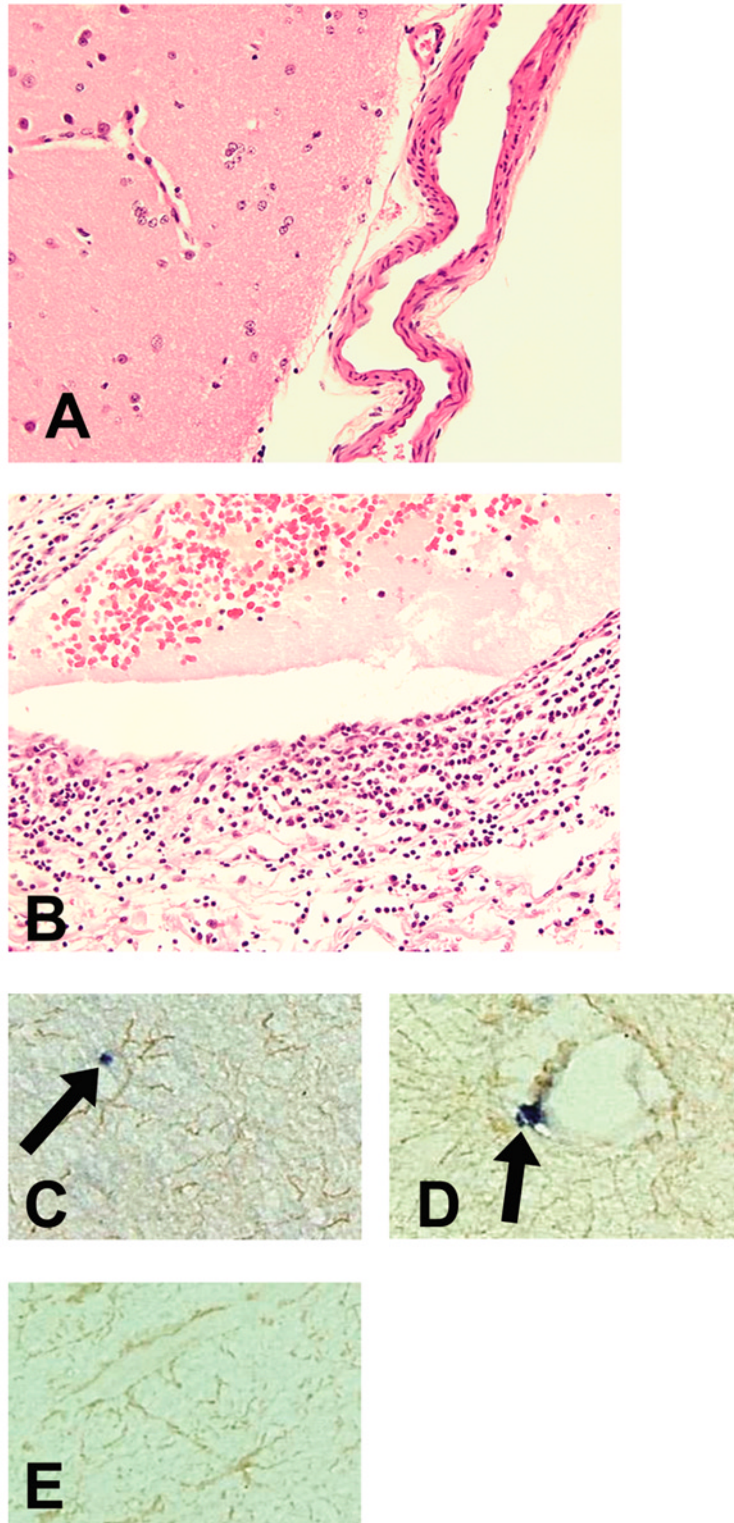


**Figure 3** Histopathological analysis and *in situ* PCR of macaques infected with either SIV *nef*/LTR-deleted DNA or SIV wild-type DNA. (**A**) Meningeal vessels of monkey 17 infected with SIV *nef*/LTR-deleted DNA showing no evidence of inflammatory cells and normal underlying cortex (×200; H&E). (**B**) Meningeal vessels of monkey 12 infected with SIV wild-type DNA showing lymphohistiocytic perivascular infiltration (×200; H&E). (**C**) *In situ* PCR to detect SIV *gag* DNA in basal ganglia sections from monkey 12 infected with SIV wild-type DNA, arrow points to SIV *gag* DNA–positive cell. (**D**) SIV *gag* DNA–positive cell located around a blood vessel. (**E**) SIV-uninfected control macaque, monkey 6, negative for SIV *gag* DNA in basal ganglia sections after *in situ* PCR (×400; NBT/BCIP product stain, background staining to detect astrocytes by GFAP immunohistochemistry, DAB–hydrogen peroxide product stain).

examined, suggesting not only a lack of neurovirulence but also diminished neurotropism.

Systemic viral load may also play an important role in the development of CNS disease. The macaques infected with *nef*-deleted virus had much lower systemic viral loads that may have resulted in diminished entry of the SIV into the brain. Zink *et al* (1997) demonstrated that macaques with the most severe neurological lesions had the highest plasma SIV, suggesting a relationship between viral load in the peripheral blood and the severity of neurological lesions.

The third requirement for the development of CNS disease is immunosuppression. Previous studies have demonstrated that SIV strains that did not have a strong immunosupressive phenotype did not induce significant neurological lesions in macaques (Mankowski *et al*, 1997; Zink *et al*, 1999). Macaques with the most severe neurological lesions had the most rapid decline, or very low absolute CD4 cell counts, demonstrating a significant relationship between the development of immunosuppression and the presence of neurological lesions and high levels of virus replication in the CNS and periphery (Zink *et al*, 1997).

We identified a reversion from *nef*/LTR to wild-type SIV in two of the monkeys infected with the SIV *nef*/LTR, M16 and M18. This was attributed to either a recombination event with positive intact 5′ LTR (M16) (Kent *et al*, 2002), or a superinfection with SIVmac251 following challenge (M18). Of note, when the *nef*/LTR-deleted SIV strain was replaced with wild-type SIV, no wild-type SIV DNA was found in the brain by extracted DNA or *in situ* PCR, despite the presence of high plasma and CSF SIV RNA levels. It is possible, at least in the short term, that the initial infection with *nef*-deleted SIV provides some protection against CNS infection, even in the presence of high levels of systemic wild-type SIV. The presence of brain inflammation and high CSF SIV RNA does suggest, however, that in humans with attenuated HIV-1 infections, there is a potential for neuropathology in the long term. Indeed, this has been observed in the Sydney Blood Bank Cohort. After a very extended follow-up (15 to 19 years), three members of the Sydney Blood Bank Cohort (including the donor) have declining CD4 counts and plasma HIV-1 RNA of $10^3$ to $10^4$ copies/ml. The donor (D36), following the onset of neurological symptoms, has been diagnosed with HIVD (Bruce Brew, personal communication). It has recently been reported that HIV-1 viral strains from some of the cohort have shown changes in the *nef*/LTR sequences over the past 5 years (Churchill *et al*, 2001). Changes in the *nef*/LTR region on viral replication have shown an evolution towards a fitter viral species. The promoter activities of the various evolving LTR sequences suggest there has been an evolution to a more transcriptionally active promoter, similar to wild-type virus. This suggests the potential for an HIV-1 attenuated virus to eventually cause neurological disease.

There are a number of limitations in this study, including the relatively small number of macaques utilized, the small number of CSF samples from each macaque, and the possibility of sequestration of SIV DNA in a compartment of the brain not analyzed in this study. Although this study examined multiple brain regions for the presence of SIV DNA by a variety of PCR-based methods, and suggested that *nef*/LTR-deleted SIV have lost their capacity to initiate CNS infection, this needs to be confirmed by a larger study.

In summary, our results suggest that *nef*/LTR-deleted SIV strains have a diminished ability to enter the CNS and appeared to provide some protection against the subsequent entry of wild-type SIV into the brain. Inoculating macaques with purified proviral plasmid SIV DNA will provide an additional tool to identify specific viral genes that may be important in neurovirulence and the development of CNS SIV infection.

## Materials and methods

*SIV plasmids*

Proviral SIV constructs were engineered with either a single deletion in the 3′ *nef*/LTR overlap region ($SIV_{sbbc\Delta3}$) analogous to the common deletion observed in HIV-1 strains isolated from the Sydney Blood Bank Cohort, or an additional identical deletion in the 5′ LTR ($SIV_{sbbc\Delta3\Delta5}$) (Figure 1), as previously described (Kent *et al*, 2001).

*Infection of macaques with SIV*

Six pigtail macaques (*M. nemestrina*, aged 10 to 16 months) were inoculated with wild-type or *nef*/LTR-deleted SIV DNA constructs ($SIV_{sbbc\Delta3}$ or $SIV_{sbbc\Delta3\Delta5}$), delivered either intramuscularly (300 g) or epidermally via gene gun (15 g) as previously described (Kent *et al*, 2001). An additional pigtail macaque (M20) inoculated with SIVmac251 virus intrarectally was also studied. Four SIV-negative macaques previously inoculated with nonpathogenic HIV-1 (Kent *et al*, 1997, 1998) were studied as controls. The studies were approved by the institutional Animal Ethics Committee. Macaques underwent clinical assessment with regular blood sample collection and were euthanized at development of manifestations of SIV infection, or the persistence of high plasma SIV RNA levels ($>10^5$ copies/ml) (Kent *et al*, 2001). Brains were removed with short postmortem delays and both frozen and fixed tissue samples collected. Tissue to be fixed was placed in 4% paraformaldehyde and embedded in paraffin. Six-micrometer sections from the mid-frontal gyrus and basal ganglia were placed on charged glass slides (Superfrost Plus, Fisher Scientific, Ontario, Canada). Hematoxylin and eosin (H&E) counterstain was performed on the fixed tissue and neuropathology examined.

*CSF and plasma SIV viral load determination*
Real-time RT-PCR of SIV *gag* RNA was utilized to determine SIV viral load in the CSF of macaques. The lower limit of detection for this assay is 1500 RNA copies/ml. Viral RNA was extracted using the RNeasy Mini Kit (QIAGEN, Victoria, Australia) according to the manufacturers protocols, and real-time RT-PCR was carried out as previously described (Jin *et al*, 1999). Plasma SIV RNA was quantified by either real-time RT-PCR or bDNA as previously described (Kent *et al*, 2001).

*DNA extraction*
DNA was extracted from samples of frozen tissue from the temporal, parietal, and frontal lobes and midbrain using the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's protocols and stored in Buffer AE (QIAGEN). DNA was extracted from PBMCs as previously described (Kent *et al*, 2001).

*Amplification of SIV* gag *by PCR*
Amplification of the SIV *gag* region, using primers F1236 (5′-TTGCAGGTAAGTGCAACACA-3′) and R1579 (5′-CTGCGTGAATGCACCAGAT-3′) (Geneworks, Adelaide, Australia) produced a 350-bp product. Amplifications were carried out in an Omnigene thermocycler (Hybaid, Middlesex, UK) and negative controls containing all the components of the reaction except the template DNA were included with each set of PCR reactions to monitor contamination. DNA samples from the four SIV-negative macaques were also included in each PCR reaction. Amplification was carried out in 50-$\mu$l reactions containing 1× Reaction Buffer (Fisher-Biotech, Perth, Australia) (670 mM Tris-HCl, pH 8.8, 166 mM $[NH_4]_2SO_4$, 4.5% Triton X-100, and 2 mg/ml gelatin), 1.25 mM $MgCl_2$, 200 $\mu$M each dNTP, 0.4 $\mu$M each primer, 1.375 units of Taq DNA polymerase (Fisher-Biotech), 6 $\mu$l DNA, and water to a final volume of 50 $\mu$l. The thermal cycling consisted of one cycle of 3 min at 94°C, 1 min at 56°C, and 30 s at 72°C, followed by 35 cycles of amplification (94°C for 30 s, 56°C for 1 min, and 72°C for 30 s), with a final extension at 72°C for 5 min.

*Amplification of SIV* nef *by nested PCR*
Nested PCR analysis of SIV *nef* DNA from tissue samples was performed using oligonucleotide primers Odp.193 (5′-AGTGCTGGAGAGAACCTCCC-3′) and Odp.239 (5′-CAGAGACTCTTGCGGGCGC-3′) for the first round and primers Odp.191 (5′-ATACTCGCAATCCCCAGG-3′) and Odp.192 (5′-TGTGGAAAGTCCCTGCTG-3′) for the second round of amplification. SIV wild-type and SIV *nef*/LTR-deleted produced 700-bp and 600-bp products, respectively. First-stage amplification was carried out in 25-$\mu$l reactions containing 1× Reaction Buffer (Fisher-Biotech) (see above), 1.5 mM $MgCl_2$, 200 $\mu$M each dNTP, 0.32 $\mu$M each primer (Odp.193 and Odp.239), 1.1 units of Taq DNA polymerase (Fisher-Biotech), 3 $\mu$l DNA, and water to a final volume of 25 $\mu$l. The thermal cycling consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 1 min 30 s, 64°C for 1 min, and 72°C for 2 min 30 s), with a final extension at 72°C for 7 min. The second-stage amplification was carried out with primers Odp.191 and Odp.192 using 3 $\mu$l of a 1-in-1000 dilution in $H_2O$ of the first-stage PCR product as a template. Reagent conditions were as above and the thermal cycling consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 1 min 30 s, 58°C for 1 min, and 72°C for 2 min 30 s), with a final extension at 72°C for 7 min. Amplification of SIV DNA from PBMCs was carried out as previously described (Kent *et al*, 2001).

*Agarose gel electrophoresis*
PCR products were electrophoresed through 1.5% (*w/v*) agarose gels containing 3 $\mu$g ethidium bromide/100 ml gel in 1 × TBE (Tris-borate EDTA). Ten microliters of product was mixed with gel-loading dye and run with a 100-bp DNA ladder (Life Technologies, Melbourne, Australia) to estimate product size. Electrophoresis was carried out at 100 V for 1 h and products were visualized using a ultraviolet transilluminator and photographed.

In situ *PCR to detect SIV* gag *DNA in the mid-frontal gyrus and basal ganglia*
*In situ* PCR was performed on multiple 6-$\mu$m serial sections of paraffin-embedded tissue from the mid-frontal gyrus and basal ganglia of each monkey to detect SIV *gag* DNA. Sections were dewaxed, hydrated in graded alcohols, and incubated in proteinase K (30 $\mu$g/ml) (Roche Diagnostics, New South Wales, Australia) at 37°C for 10 min. Following phosphate-buffered saline (PBS) washes, the tissue was postfixed in 4% paraformaldehyde for 5 min, and acetylated twice with 0.25% acetic anhydride in 0.1 M triethanolamine. Following washes and dehydration, 125 $\mu$l of a PCR mix containing 700 $\mu$M of dNTP, 30 pmol of each SIV *gag* primer (F1236 [5′-TTGCAGGTAAGTGCAACACA-3′] and R1579 [5′-CTGCGTGAATGCACCAGAT-3′]; Geneworks), PCR buffer (3.5 mM magnesium chloride [$MgCl_2$], 10 mM Tris–hydrochloric acid [HCl], 50 mM potassium chloride [KCl], 0.1 mg of gelatin/ml; pH 8.3 at 20°C), and 10 units of Taq DNA polymerase (Roche Diagnostics, New South Wales, Australia) was heated to 75°C and added to each slide preheated to 55°C. "Gene Frame II" coverslips (Advanced Biotechnologies, Surrey, UK) were used to encase the specimen area to eliminate evaporative loss of reagents during the PCR–*in situ* hybridization. The PCR was performed for 35 cycles (94°C for 30 s; 55°C for 1 min, and 72°C for 30 s) in a Hybaid Omnislide thermocycler (Hybaid). The coverslips were removed and *in situ* hybridization

performed with a cocktail of two digoxigenin end-labeled oligonucleotide probes (SIVGAG223 [5′-TTTCTAATTCATCTGCTTTITTCCCT-3′], SIV-GAG219 [5′-GTATTAIAAAGGCTTTTTAAATTTTC-TGA-3′], I = inosine [Geneworks]) internal to the PCR primer pair. Following washes, the sections were incubated for 3 h at room temperature with an alkaline phosphatase–labeled anti-digoxigenin antibody (1:500; Roche Diagnostics). The blue reaction product was revealed with nitroblue tetrazolium/bromochloroindolyl phosphate (Roche Diagnostics). Control samples were included in each run of PCR–*in situ* hybridization. Sections from the same tissue blocks were treated without Taq DNA polymerase or with irrelevant primer pairs. In addition, sections without preceding PCR and/or sections treated with irrelevant oligonucleotide probes were used. Tissue sections from a SIV-negative macaque was also included in each run.

*Astrocyte glial fibrillary acidic protein (GFAP) immunohistochemistry*

GFAP antibody immunohistochemistry, for the detection of astrocytes, was performed as a background stain on the sections immediately after *in situ* PCR. A rabbit anti-GFAP antibody (Zymed, San Francisco, CA) was used with the avidin-biotin complex method and a diaminobenzidine (DAB) hydrogen peroxide product as the colorimetric substrate.

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