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Simian immunodeficiency virus mac251 infection of astrocytes

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Human immunodeficiency virus type 1 (HIV-1) infection of astrocytes has been demonstrated in the brains of patients with AIDS dementia complex (ADC) and may play an important role in neuropathological pathways of HIV-related encephalopathy. SIVmac-infected monkeys develop an acquired immunodeficiency syndrome (AIDS) with CNS involvement which is quite similar to that seen in human AIDS. We investigated the *in vitro* infection of primary astrocytes derived from adult macaques with SIVmac251 or an isogenic virus that expresses a non-functional Nef protein (SIVmac251-ΔNef). In both cases we observed that viral expression was mostly limited to early regulatory genes after a transient phase of late viral gene expression (i.e. *env* and *gag*), as reported for HIV-1-infected astrocytes *in vivo*. Late viral gene expression could be reactivated by TNF-α, GM-CSF and IFN-γ treatment of SIVmac251-infected astrocytes but not by similarly treated SIVmac251-ΔNef-infected cells. Our findings suggest that Nef is not involved in the restricted expression of SIV in astrocytes, but may be important for astrocytes to function as a viral reservoir in the CNS. In additional experiments, we demonstrated Rev and Nef expression in 17 of 27 primary astrocyte cultures derived from macaques infected by SIVmac251. Nef was located in the cytoplasm of astrocytes infected by SIVmac251 *in vivo*, but displayed perinuclear localisation after infection *in vitro*. Attempts to activate late viral gene expression by astrocytes infected *in vivo* using cytokines or by coculture with human cord blood mononuclear cells were unsuccessful. *Journal of NeuroVirology* (2000) **6,** 173–186.

Keywords: primary astrocytes; macaques; SIV mac

Introduction

AIDS patients frequently develop neurological disorders that result from HIV replication in the brain (Navia and Price, 1987). Clinical manifestations vary from mild cognitive and motor impairments to acute dementia and paralysis (Navia *et al*, 1986a,b; Price *et al*, 1988; Gray *et al*, 1991). The neuropathological hallmarks of HIV encephalitis (HIVE) include cerebral atrophy, macrophage infiltration, multinucleated giant cells, microgliosis, astrogliosis, widespread myelin pallor and neuronal loss (Navia *et al*, 1986a,b; Gray *et al*, 1996). The neuropathogenic pathways underlying HIVE are unclear but appear to be multifactorial (Epstein and Gendelman, 1993). Although HIV infection of neurons and oligodendrocytes has occasionally been reported (Stoler *et al*, 1986; Gyorkey *et al*, 1987; Harouse *et al*, 1991a,b; Harouse and Gonzalez-Scarano, 1996; Nuovo *et al*, 1994; Bagasra *et al*, 1996), neuropathogenic mechanisms are more likely to involve the release of neurotoxic factors such as quinolinic acid, platelet activating factor, eicosanoides, tumour necrosis factor (TNF)-α, and nitric oxide into the CNS by HIV-infected microglia and/or infiltrated macrophages. Indeed, these cells are the principal HIV targets in the CNS and the only types that can support productive HIV replication (Navia *et al*, 1986a,b; Koenig *et al*, 1986; Price *et al*, 1988; Kure *et al*, 1991).

Astrocyte infection consistently occurs in a small percentage of cells in brain tissues from both paediatric and adult AIDS patients, and on occasion

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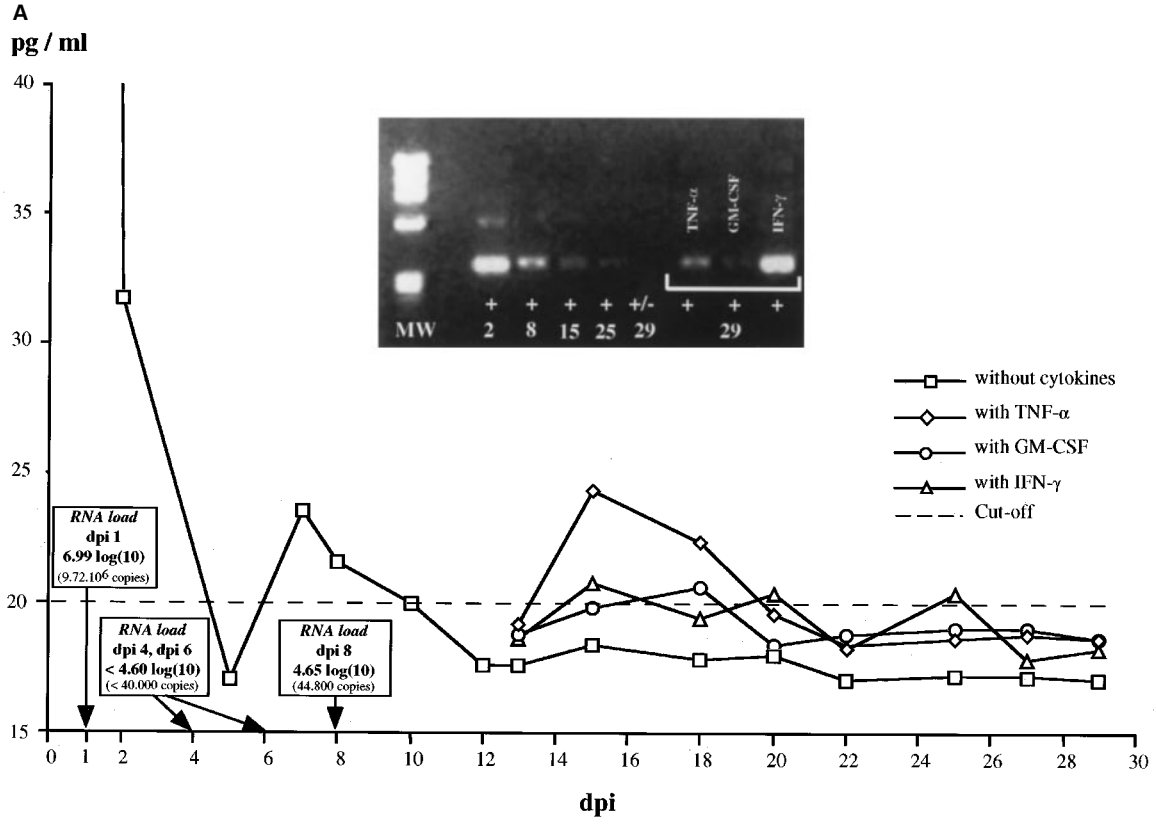
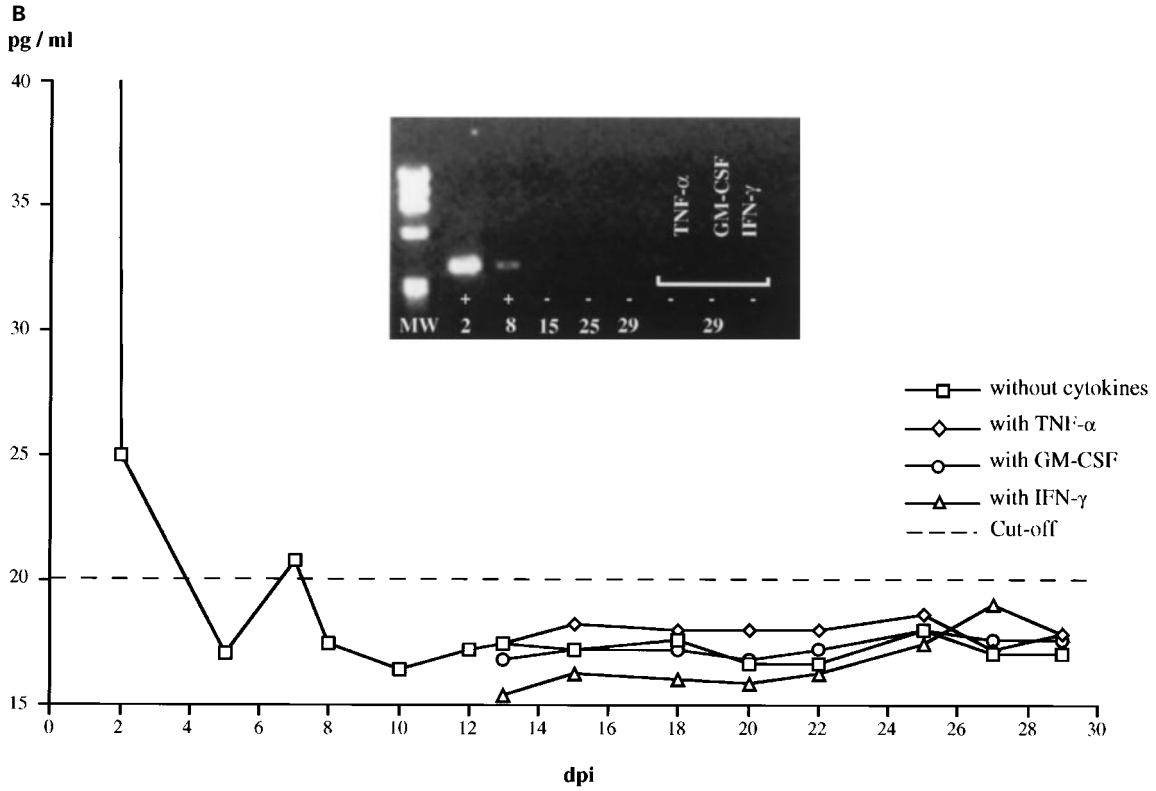
correlates with the presence of HIVE. However, infection of astrocyte by HIV *in vivo* is largely restricted to the expression of the viral regulatory proteins Nef and Rev (Brack-Werner *et al*, 1992;





Tornatore *et al*, 1994a,b; Kleinschmidt *et al*, 1994; Saito *et al*, 1994; Ranki *et al*, 1995). A similar pattern of restricted infection has also been observed *in vitro* after HIV infection of astrocytoma cell lines (Cheng-Mayer *et al*, 1987; Brack-Werner *et al*, 1992, 1997; Kleinschmidt *et al*, 1994) or primary foetal astrocytes (Tornatore *et al*, 1991, 1994b; Nath *et al*, 1995, 1996; Canki *et al*, 1997; Brengel-Pesce *et al*, 1997). The proposed mechanisms underlying the restricted astrocyte infection have been linked to the overexpression of Nef (Kohleisen *et al*, 1992; Brack-Werner *et al*, 1992; Tornatore *et al*, 1994b; Fiala *et al*, 1996; Gorry *et al*, 1998), an alteration of Rev function (van Empel *et al*, 1999; Neumann *et al*, 1995) or to an astrocyte-specific suppression of HIV mRNA translation (Gorry *et al*, 1999). Furthermore, HIV appears to be non-cytopathic in astrocytes, and productive infection may be reactivated by stimulation with TNF-α or interleukin-1β (IL-1-β) (Tornatore *et al*, 1991; Swingler *et al*, 1992; Klein *et al*, 1999), suggesting that astrocytes infected *in vivo* may serve as viral reservoirs in the brain (reviewed by Brack-Werner, 1999). Astrocytes are the predominant cell type in the brain, so even the small percentage of astrocytes infected *in vivo* may constitute a significant pool for the production of new virus particles. Since primary human astrocytes do not express CD4 (Ma *et al*, 1994; Nath *et al*, 1996) the principal HIV receptor on astrocytes remains to be determined. However, human and simian astrocytes express the major HIV and SIV chemokine co-receptors. CXCR4 and CCR5, as well as orphan receptors BOB, BONZO and Apj (Klein *et al*, 1999; Croitoru *et al*, 1998; Oh *et al*, 1999). It is unclear to what extent chemokine co-receptors and orphan receptors mediate virus entry into astrocytes. Moreover, another receptor able to bind gp120 associated with an endocytic pathway has been recently described on astrocytes (Hao and Lyman, 1999).

Astrocytes have important regulatory roles that are essential for normal brain function. They provide trophic and nutrient support for neurons, and are involved in detoxification of the brain, the maintenance of the blood brain barrier and the local immune response in the CNS (Eddelston and Mucke, 1993). The precise consequences of astrocyte infection by HIV remain unknown, but may include interference with their normal functions as well as a direct role in neurotoxicity by the release of neurotoxic factors in the CNS.

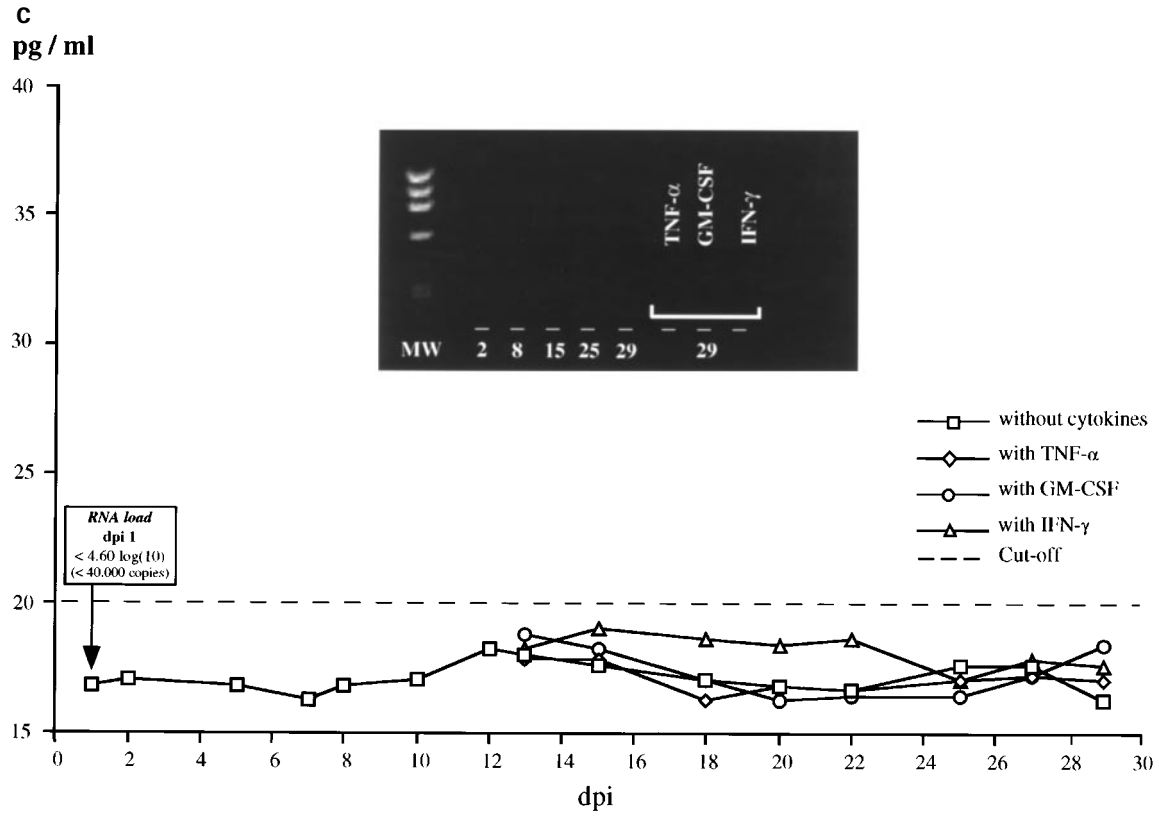


Figure 1 The SIVmac p27 protein has been quantified in supernatants from astrocyte cultures infected with SIVmac251 (**A**), infected with SIVmac251-ΔNef (**B**), uninfected (**C**). All cultures were treated with cytokines TNF-α, IFN-γ and GMCSF at 13 days post infection. All experiment were stopped at 29 days post infection. The baseline of significance for these experiments was fixed at 20 pg of p27/ml. SIV RNA levels in culture supernatants were evaluated using the branched-DNA technique (Chiron) and Proviral DNA detection by Polymerase Chain Reaction.

The simian immunodeficiency virus mac251 (SIVmac251) causes pathological changes and clinical diseases in macaques that are strikingly similar to human AIDS, but with a relatively accelerated time scale of disease. Moreover, infected macaques may develop an encephalopathy (SIVE) which is consistent with the neuropathological hallmarks in human HIVE (Ringler *et al*, 1988; Desrosiers, 1990; Zink *et al*, 1998). Thus, SIV-infected macaques provide a relevant model to evaluate neuropathogenic pathways of HIV infection in the brain (Sharer *et al*, 1988; Axthelm and Shiigi, 1990; Brinkmann *et al*, 1993; Clements *et al*, 1994; Vitkovic *et al*, 1995; Sasseville and Lackner, 1997; Fox *et al*, 1997; Rausch *et al*, 1999).

To date, simian astrocyte infection by SIVmac251 has not been addressed. In this study, we demonstrated that SIVmac251 can infect primary astrocytes derived from adult macaque brains. We also describe the detection of astrocytes infected by SIVmac251 *in vivo* in primary astrocyte cultures derived from SIVmac251-infected macaques. Our study contributes to better understanding neuropathgenic mechanisms of HIV, and demonstrates for the first time an *ex vivo* system that allows the study of *in vivo*-infected brain-derived cells.

Results

SIVmac251 infection of primary simian astrocyte cultures

One day post-infection (p.i.), viral RNA reached at 6.99 log(10) (equivalent to 9.72 10^6 of copies) in *in vitro* infected primary simian astrocyte cultures coinciding with a very high amount of p27. Proviral SIVmac251 DNA was detected using nested-PCR, 2 days p.i., which coincided with the detection of p27 release in cell culture supernatants (Figure 1A).

We then observed a decrease in both p27 concentration in culture supernatants 5 days p.i. and level of viral RNA which was under the threshold of positivity of the assay (4.60 log(10) equivalent to $<40.10^3$ of copies) at days 4 and 6. Then, a weak but significant p27 release (comprising between 20 and 25 pg/ml) correlated with an increase of viral RNA level (4.65 log(10) equivalent to 44.8 10^3 of copies) was detected at days 7 and 8 p.i..

After day 8 p.i., no p27 could be detected in cell culture supernatants, and the concentration of proviral DNA decreased progressively. However, viral sequences were still detectable 29 days p.i.. Transient detection of p27 release at day 8 p.i. coincided with the detection of *env* gene products in 0.05 to 0.1% of GFAP-positive cells by immunocytochemistry (Figure 2B) confirming both the astrocyte infection and the late gene expression by infected cells at that time. In contrast, expression of the regulatory proteins Rev and Nef was not evident at day 8 p.i., but was detectable at day 15 p.i. in approximately 0.2 to 0.4% astrocytes (Figure 2C,D) and was persistent up to day 29 p.i. Conversely, Env was not detectable during the persistent expression of Rev and Nef.

Nef detected by immunocytochemistry displayed a typical perinuclear localization, whereas Rev was localized mainly in the cytoplasm and not observed in the nucleus. Perinuclear expression of Vpx was also detected in rare astrocytes at days 15 and 25 p.i. (Figure 2A). It is important to note that the infected astrocytes did not differ morphologically to the uninfected cells.

SIVmac251-ΔNef infection of primary simian astrocyte cultures

SIVmac251-ΔNef infection of primary macaque astrocyte cultures resulted in similar replication kinetics to those observed after SIVmac251 infection (Figure 1B). However, SIVmac251-ΔNef proviral DNA was detected only at days 2 and 6 p.i. and not at the later timepoints, suggesting a more significant loss of infected cells compared to that observed in SIVmac251-infected cultures. This was further confirmed by immunofluorescence studies, which demonstrated Rev in infected astrocytes at days 15 and 25 (in less than 0.1% of astrocytes) but not at day 29. Immunostaining performed with antibodies against Nef, Vpx and Env remained negative at all timepoints.

TNF-α, GM-CSF and IFN-γ enhancement of SIVmac251 but not SIVmac251-ΔNef late gene expression in in vitro *infected primary macaque astrocytes*

At 13 days p.i., treatment of SIVmac251-infected primary macaque astrocytes with TNF-α, GM-CSF or IFN-γ led to a weak but significant stimulation of p27 release (Figure 1A), but no effect was observed when cells were similarly treated at day 29 p.i. (data not shown). The effects of TNF-α and GM-CSF stimulation were transient, with the peaks of p27 release at days 2 and 5 post-stimulation, respectively. IFN-γ resulted in p27 release that was detectable as early as 2 days after treatment but also up to 12 days post-stimulation. Furthermore, TNF-α and IFN-γ but not GM-CSF led to an increase in proviral DNA synthesis at day 29 p.i. compared to untreated cultures (Figure 1C). This effect was particularly marked for IFN-γ. Similar experiments were performed on SIVmac251-ΔNef-infected primary macaque astrocytes but neither TNF-α, GM-CSF, nor IFN-γ were able to restimulate detectable p27 release in cell culture supernatants or increase the DNA proviral load in astrocyte cultures compared to untreated controls (Figure 1B).

SIVmac251 proviral DNA detection in primary astrocyte cultures derived from infected macaques

Primary astrocytes from the brains of 29 macaques infected with SIVmac251 and/or HIV-2ROD were isolated and cultured as described in Materials and

methods (Table 1). Twenty-seven of the macaques were infected with SIVmac251 for 0.1–3 years. Among them, six had been infected 7 years prior with HIV-2ROD. Two additional monkeys were infected with HIV-2ROD alone for 1 year. None of the macaques had developed significant clinical sign of SIVE at the time of sacrifice, but six of them presented with an AIDS-like disease. CSF samples were negative for p27 antigen, but SIVmac251 genomic RNA was detected by RT–PCR in samples from three macaques without any correlation with the clinical status, the plasma viremia, or the blood T lymphocyte CD4/CD8 ratio.

Detection of proviral DNA by nested-PCR was usually performed in triplicate using DNA samples from three different primary astrocyte cultures that were extracted at 30, 45 and 60 days after seeding. In astrocyte cultures derived from eight monkeys SIVmac251 proviral DNA was detected in all three cultures, and was detected at least once in cultures from 17 monkeys (Table 1). Astrocyte cultures from 11 monkeys were consistently negative for proviral

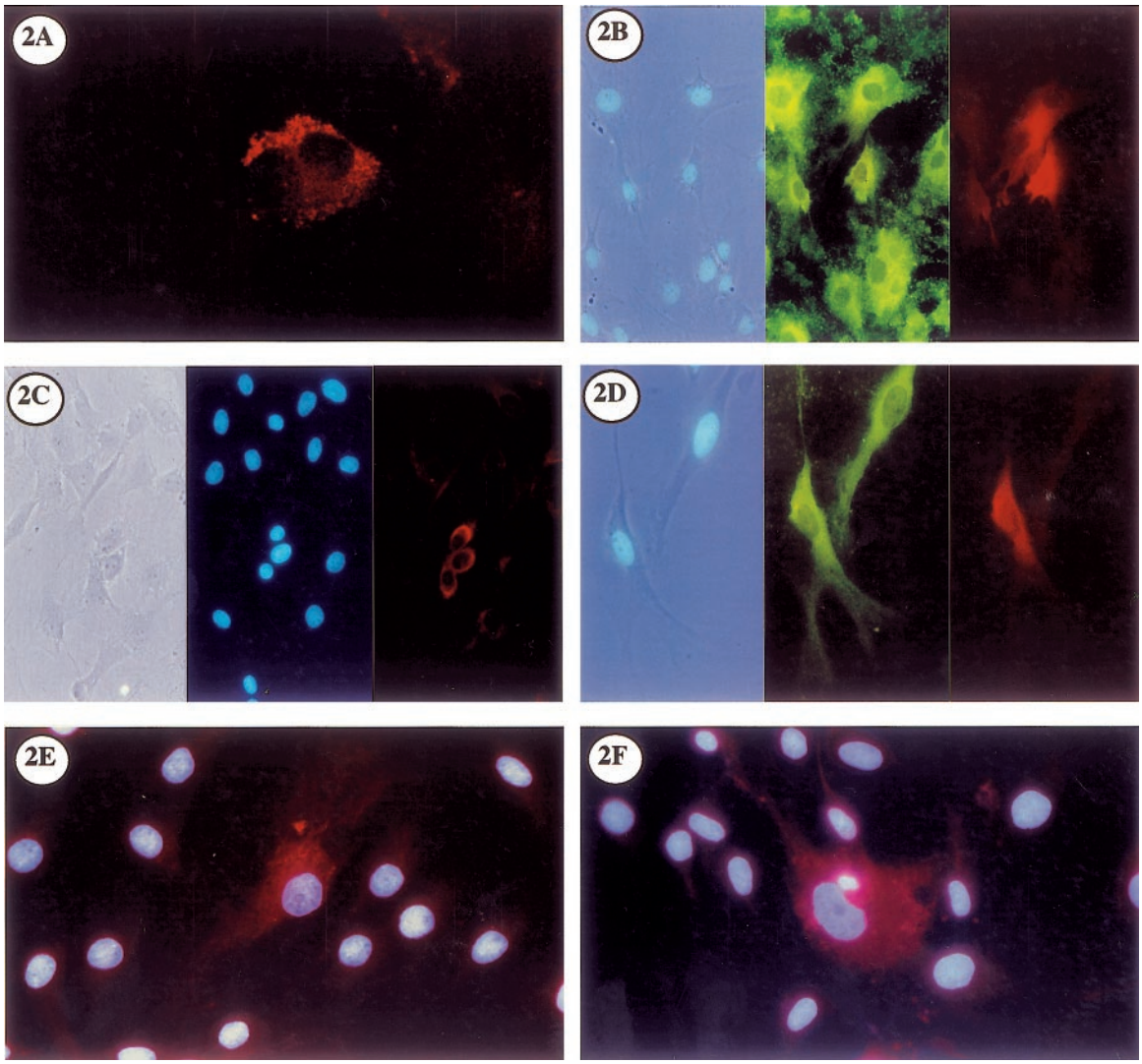


Figure 2 Fluorescence photographs of immunocytochemical detection of SIVmac proteins in *ex vivo* and *in vitro* infected-astrocytes in primary cultures: combinations of a single, dual or triple labelling with mAbs against SIVmac251 proteins secondary stained with goat anti-mouse IgG_1 antibodies conjugated with Texas Red; polyclonal antisera against GFAP (Sigma), and then with goat anti-rabbit antibodies conjugated with FITC; and nuclear staining with DAPI have been used. (**A**, **C**, **D**) were performed with 15 days p.i. *in vitro* SIVmac251-infected astroglial cultures and (**B**) with 7 days p.i. *in vitro* SIVmac251-infected astroglial cultures. (**E** and **F**) corresponding to stainings performed with *ex vivo* SIVmac251-infected astroglial cultures 35 days after seeding. (**A**) Labelling with mAb against Vpx, then with goat anti murin IgG1 antibody conjugated with Texas Red; magnification, ×600. (**B**) Triple labelling with mAb against gp120/160, antisera against GFAP (FITC) and nuclear staining with DAPI; magnification, ×400. (**C**) Dual labelling with mAb against Nef and nuclear staining with DAPI; magnification, ×400. (**D**) Triple labelling with mAb against Rev (Texas Red), antisera against GFAP (FITC) and nuclear staining with DAPI; magnification, ×400. (**E**) Dual labelling with mAb against Nef and nuclear staining with DAPI (1 μg/ml); magnification, ×600. (**F**) Dual labelling with mAb against Rev and nuclear staining with DAPI; magnification, ×600.

Table 1

Labels	*Species*	*Virus*	*Years PI*	*Infection route*	*Clinical signs*	*Plasmatic viremia p27*	*Cellular viremia TCID*	*CD4/CD8*	*SIV ARN on CSFs (RT–PCR)*	*Pro viral detection on astrocytes SIV mac 251 30 d**	*45 d*	*60 d*	*HIV-2 30 d*	*45 d*
C907	Cynomolgus	SIV mac 251	1	IV	–	ND	10	0	–	+	+	+	ND	ND
C 388	Cynomolgus	SIV mac 251	1	IV	–	ND	65	0.2	–	+	+	–	ND	ND
C 305	Cynomolgus	SIV mac 251	1	IV	–	ND	65	0.7	–	–	–	–	ND	ND
C 307	Cynomolgus	SIV mac 251	1	IV	–	ND	3	2.1	–	+	+	+	ND	ND
B 713	Cynomolgus	SIV mac 251	1	IV	–	ND	195	0.17	–	–	–	–	ND	ND
A 959	Cynomolgus	SIV mac 251	1	IV	–	ND	1	0.4	+	–	–	–	ND	ND
46880	Cynomolgus	SIV mac 251	1	IV	–	ND	1	0.87	–	+	+	+	ND	ND
46713	Cynomolgus	SIV mac 251	1	IV	–	ND	3	0.3	–	–	–	+	ND	ND
H 383	Cynomolgus	SIV mac 251	1	IV	–	ND	2	0.85	–	+	+	–	ND	ND
I 823	Cynomolgus	SIV mac 251	1	IV	–	ND	1	0.76	–	+	+	–	ND	ND
46702	Cynomolgus	SIV mac 251	1	IV	–	ND	22	0.6	–	+	–	–	ND	ND
13 B	Cynomolgus	SIV mac 251	1	IV	–	ND	1	0.5	+	+	–	–	ND	ND
Pat	Cynomolgus	SIV mac 251	1	IV	–	0.04	1	0.28	–	+	–	–	ND	ND
49683	Cynomolgus	SIV mac 251	0.5	Vaginal	AIDS like disease	0.03	1	1	–	+	+	+	ND	ND
I872A	Cynomolgus	SIV mac 251	0.5	Vaginal	AIDS like disease	0	1	ND	–	–	–	–	ND	ND
49094	Cynomolgus	SIV mac 251	1	IV	AIDS like disease	0.251	2	0.19	–	–	–	–	ND	ND
86757	Cynomolgus	SIV mac 251	3	IV	–	0.05	45	0.12	–	–	ND	ND	ND	ND
49101	Rhesus	SIV mac 251	3	IV	–	ND	ND	ND	–	+	+	+	ND	ND
49103	Rhesus	SIV mac 251	3	IV	–	ND	ND	ND	–	+	+	+	ND	ND
49836	Rhesus	SIV mac 251	1	IR	AIDS like disease	ND	2	0.13	–	–	–	–	ND	ND
49778	Rhesus	SIV mac 251	1	IR	–	ND	2	0.5	–	–	+	–	ND	ND
33219	Rhesus	HIV-2+SIV	7+1	IV+IR	AIDS like disease	ND	7	0.4	+	–	–	–	–	–
33209	Rhesus	HIV-2+SIV	7+1	IV+IR	AIDS like disease	ND	1	0.2	–	–	+	+	–	–
34771	Rhesus	HIV-2+SIV	7+1	IV+IR	–	ND	2	0.3	–	–	–	–	–	–
34433	Rhesus	HIV-2+SIV	7+1	IV+IR	–	ND	1	1.02	–	+	+	+	–	–
34782	Rhesus	HIV-2+SIV	7+0.1	IV+IR	–	0.09	94	0.1	–	+	+	+	+	+
37202	Rhesus	HIV-2+SIV	7+1	IV+IR	–	ND	1	0.11	–	ND	–	–	–	–
H62	Cynomolgus	HIV-2	1	IV	Lypmhoma	ND	ND	0.1	–	–	ND	ND	+	+
G286	Cynomolgus	HIV-2	1	IV	Tuberculosis	ND	ND	0.1	–	–	–	ND	–	–
Neg 1	Cynomolgus	–	–	–	–	0	0	0	–	–	ND	ND	–	ND
Neg 2	Cynomolgus	–	–	–	–	0	0	0	–	–	ND	ND	–	ND
Neg 3	Cynomolgus	–	–	–	–	ND	ND	ND	ND	–	ND	ND	ND	ND
Neg 4	Rhesus	–	–	–	–	0	0	0	–	–	ND	ND	–	ND
Neg 5	Rhesus	–	–	–	–	0	0	0	–	–	ND	ND	–	ND

PI, post infection; IV, intravenous; IR, intrarectal; ND, not determined; *Days after seeding of primary astroglial cultures. References regarding these animals: Le Grand *et al.* (1995) and Wakrim *et al* (1996).

DNA. The detection of both SIVmac251 and HIV-2ROD sequences was achieved in one of six doubly-infected monkeys, and HIV-2 proviral DNA was detected in one of two HIV-2ROD mono-infected monkeys. As controls, nested-PCR reactions performed to amplify either SIVmac251 or HIV-2 proviral sequences on DNA extracts from primary astrocyte cultures derived from five uninfected macaques were always negative (data not shown). However, p27 release was not detected when any of the infected astrocyte cultures were stimulated with TNF-α, GM-CSF or IFN-γ, or by coculture with human cord blood mononuclear cells (data not shown).

Immunocytochemical studies performed on primary astrocyte cultures derived from four monkeys (34433, 49778, 13B, 49101) at 30 and 45 days after seeding confirmed astrocyte infection by the detection of Nef (Figure 2E) and Rev (Figure 2F) in 0.05% to 0.1% of GFAP-positive cells. However, in contrast to the perinuclear localization of Nef observed in the *in vitro* SIVmac251-infected primary astrocytes, Nef staining was consistently diffuse and cytoplasmic in the primary astrocyte cultures derived from infected monkeys. Rev, however, was predominantly localized in the astrocyte cytoplasm under both infection conditions.

Astrocytes expressing Env or Vpx were not detected in the primary cultures from infected monkeys, confirming the lack of expression of SIV late genes during restricted SIV infection of astrocytes *in vivo*. Finally, no correlation could be drawn between astrocyte infection in the *ex vivo* primary cultures and the clinical status, plasma viremia nor blood lymphocyte T4/T8 ratio.

Discussion

In the central nervous system, both HIV-1 and SIV have been shown to replicate preferentially in macrophages, microglia and MNGC (Wiley *et al*, 1986; Koenig *et al*, 1986; Vazeux *et al*, 1987; Michaels *et al*, 1988; Price *et al*, 1988; Kure *et al*, 1990; Lackner *et al*, 1991; Brinkmann *et al*, 1993; Sharer *et al*, 1988, 1996; Sharer, 1994). However, analysis of post-mortem brain tissues with combined sensitive detection methods such as *in situ*-hybridization, immunohistochemistry, *in situ*-PCR and RT–PCR have recently demonstrated astrocyte infection as a frequent event in AIDS encephalopathy (Tornatore *et al*, 1994a,b; Saito *et al*, 1994; Ranki *et al*, 1995; Sharer *et al*, 1996; Bagasra *et al*, 1996). These studies demonstrated that HIV expression in astrocytes is mostly limited to the regulatory genes *rev* and *nef*. A similar restricted infection phenotype has been observed upon HIV infection of astrocyte cell lines (Cheng-Mayer *et al*, 1987; Bachelerie *et al*, 1990; Kohleisen *et al*, 1992; Brack-Werner *et al*, 1992; Kleinschmidt *et al*, 1994), and foetal astrocyte cultures (Christofinis *et al*, 1987; Tornatore *et al*, 1991, 1994b; Nath *et al*, 1995; Fiala *et al*, 1996; Canki *et al*, 1997; Bencheikh *et al*, 1999). In contrast, isolation of infected astrocytes from infected individuals has not been described, and to our knowledge, no information is available on the susceptibility of simian astrocytes to SIVmac251 infection. In the present study we have demonstrated that SIVmac251 can efficiently infect primary simian astrocyte cultures, and that infected astrocytes can be cultured directly from SIVmac251-infected adult monkeys with and without encephalopathy.

Our findings in SIVmac251-infected primary simian astrocyte cultures are in accordance with other models of astrocyte infection by HIV-1 (Tornatore *et al*, 1991; Nath *et al*, 1995; Brengel-Pesce *et al*, 1997; Bencheikh *et al*, 1999). The levels of p27 production in our virus-infected astrocytes was approximately 50-fold lower than that observed in astrocytes transfected with proviral plasmids, however the kinetics of virus production over time were similar (Bencheikh *et al*, 1999; Sabri *et al*, 1999). After a transient low-level expression of structural genes following infection (Figures 1A and 2B), viral expression was mostly limited to the regulatory viral gene products Rev and Nef.

The mechanism for the restricted viral expression in astrocytes remains poorly elucidated, but well studied. Recent reports have demonstrated that in astrocytes, Rev protein is efficiently synthesised (Gorry *et al*, 1999) and retains its ability to bind its RRE target (van Empel *et al*, 1999). However, results presented in the preceding experiments, and published by others (Brack-Werner *et al*, 1992; Brack-Werner, 1999; Kleinschmidt *et al*, 1994; Gory *et al*, 1998, 1999), demonstrated an aberrant cytoplasmic Rev localisation suggesting that the restricted infection may be associated with altered Rev function. Nevertheless, a more recent study demonstrated efficient Rev function, but inefficient translation of Rev-dependent HIV mRNA's in astrocytes transfected with proviral plasmids (Gorry *et al*, 1999).

HIV Nef protein enhances the expression of early viral genes and promotes virus infectivity in astrocytes (Bencheick *et al*, 1999). However, as for *gag* and *env*, *nef* mRNA is correctly transported to the cytoplasm of astrocytes but inefficiently translated (Gorry *et al*, 1999). In addition, the *nef* gene, but not the protein, exerts a suppressive effect on viral replication in astrocytes (Gorry *et al*, 1998). We compared infection of primary simian astrocytes with either SIVmac251 or SIVmac251-ΔNef and demonstrated that Nef is not likely to be directly involved in the restricted expression of virus. However, the inefficiency of cytokines to reactivate the expression of late viral genes in SIVmac251-ΔNef infected astrocytes indicates that Nef may be

necessary for astrocytes to function as a persistent viral reservoir.

Several cytokines are expressed at high levels in the CNS during AIDS (Benveniste, 1992, 1994; Tyor *et al*, 1992), and include IL-1β, TNF-α, IL-6, IFN-γ, GM-CSF and TGF-β. These cytokines may contribute to intracerebral inflammatory responses, as well as the enhancement of HIV-1 expression in the CNS. The capacity of TNF-α to stimulate HIV or SIV replication has been described in various cell types (Walsh *et al*, 1991; Lairmore *et al*, 1991; Kleinschmidt *et al*, 1994). In accordance with previous results obtained in primary and transformed astrocytes (Tornatore, 1991, 1994b; Swingler *et al*, 1992, 1994; Sabri *et al*, 1999), we demonstrated reactivation of SIVmac251 virus expression in primary simian astrocytes following treatment with TNF-α. Thus, TNF-α could restimulate the restricted viral expression in astrocytes *in vivo*.

The effects of IFN-γ on viral expression in infected cells are unclear. IFN-γ has been shown to decrease SIV production in primary macrophage cultures (Walsh *et al*, 1991), and inhibit HIV replication in primary and transformed astrocytes (Tornatore *et al*, 1991; Kleinschmidt *et al*, 1994). Our finding that IFN-γ enhances SIV expression in simian astrocytes is in accordance with previous findings in primary murine astrocytes (Swingler *et al*, 1992). However, the effect of IFN-γ on p27 production was less marked than TNF-α, but evident at later times after infection indicating a persistent effect. The increase in proviral copy number in IFN-γ-treated cells may be due to the ability of IFN-γ to induce chemokine receptor expression on foetal human and adult simian astrocytes (Croitoru *et al*, 1998; Klein *et al*, 1999; Oh *et al*, 1999), thus possibly rendering them more susceptible to infection.

The capacity of GM-CSF to stimulate viral expression in myeloid cells remains controversial, but appears to depend on the cell type and activation state (Folks *et al*, 1987; Perno *et al*, 1989; Walsh *et al*, 1991; Matsuda *et al*, 1995). In the present study, we demonstrated that GM-CSF is able to stimulate a weak, but significant, expression of SIVmac251 in primary adult simian astrocytes infected *in vitro*. The discrepancy between GM-CSF-mediated viral gene expression in simian astrocytes and the failure of this effect in foetal human astrocytes (Tornatore *et al*, 1991) may be due to differences in the differentiation state between adult and foetal cells, or may be explained by the ability of GM-CSF to induce proliferation of adult simian astrocytes (Guillemin *et al*, 1996).

We demonstrated that infection of simian astrocytes with SIVmac251 *in vitro* led to virus production in only a small percentage of cells. This low number of productively infected cells may be explained by: (1) a selective infection of a particular subpopulation of astrocytes, since astrocytes are known to be highly heterogenous *in vivo* (Wilkin and Levi, 1986; Wilkin *et al*, 1990; Prochiantz and Mallat, 1988; Bailey and Shippley, 1993), (2) the dependence on the activation state of the cells for susceptibility to infection. However, we did not find any increased susceptibility of astrocytes to infection when pre-treated with GM-CSF or TNF-α (data not shown), (3) a suppression in translation of viral proteins in the infected cells, which has recently been demonstrated in human astrocytes transfected with HIV plasmids (Gorry *et al*, 1999), or (4) a rapid induction of cell death in the productively infected cell population, possibly by an apoptotic pathway. The latter hypothesis is supported by our observation of high-level astrocyte death when using high viral doses for *in vitro* infections, the constant decrease in proviral load in the infected cultures, and by previous reports that demonstrate HIV-induced astrocyte apoptosis *in vitro* (Adamson *et al*, 1996) and *in vivo* (Petito and Roberts, 1995), the surface expression of Fas ligand, and Fas-mediated apoptosis in astrocytes (Saas *et al*, 1997). IFN-γ has been shown to prevent TCR-mediated apoptosis in peripheral blood mononuclear cells from infected patients (Clerici *et al*, 1994). Thus, IFN-γ may also prevent apoptosis in astrocytes infected *in vitro*, and lead to the increase in the proviral copy number that we observed.

We detected infected astrocytes in primary cultures derived from SIVmac251-infected monkeys that did not develop clinical symptoms of SIVE. One possible reason could be that the astrocytes were infected during the initial isolation steps by productively infected microglial cells. However, the very low proportion of microglial cells in the culture at this early time of culture associated with the fact we did not detect p27 release, the astrocytes were more likely to have been infected *in vivo*. In addition, some studies have shown that astrocytes are more susceptible to infection by lymphotropic viral strains compared to macrophage tropic strains that replicate in microglia (Nath *et al*, 1995; Canki *et al*, 1997; McCarthy *et al*, 1998). Comparison of proviral DNA sequences between different tissues may assist to confirm this hypothesis.

Likewise, sequencing *nef* may reveal the reason for its cytoplasmic localisation in the astrocytes infected *in vivo* compared to the typical perinuclear localisation observed in astrocytes infected *in vitro*. Functional Nef has been shown to be myristoylated and anchored to the nuclear membrane (Kohleisen *et al*, 1992; Chowers *et al*, 1994; Nath *et al*, 1995). Proviruses detected in astrocytes infected by SIVmac251 *in vivo* may have some particular altered Nef functions, as previously observed for Gag (Canki *et al*, 1997). Indeed, a functional Nef may result in more efficient viral gene expression and cell activation, and thus could be responsible for the increased cell death and progressive

disappearance of infected cells that we observed upon *in vitro* infection of astrocytes.

Our findings, together with previous reports that Nef can induce astrocyte apoptosis via transactivation of the c-kit protooncogene (He *et al*, 1997), inhibit AP-1 activation (Richard *et al*, 1997), inhibit extracellular signal-regulated kinase-dependent DNA synthesis (Romero *et al*, 1998), suppress productive HIV infection in astrocytes (Gorry *et al*, 1998), and mediate chemotaxis (Koedel *et al*, 1999), all indicate that Nef or its gene may be involved in the regulation of HIV replication in astrocytes and probably in HIV neuropathogenesis. The exact consequences of HIV infection of astrocytes remain unknown, but likely to be important in the neuropathogenic pathways of HIVE.

Materials and methods

Animals

Twelve adult Rhesus macaques (*Macaca mulatta*) and 22 cynomolgus macaques (*Macaca fascicularis*) were involved in this study. Twenty-nine of them were infected with SIVmac251 and/or HIV-2ROD (Table 1, Le Grand *et al*, 1995; Wakrim *et al*, 1996). Infected monkeys were remained in single cages housed in biosafety level 3 facilities in accordance with EEC guidelines for primate experiments (86/609/EEC, Journal Officiel des Communautés Européennes, L358, December 18, 1986). They were always handled under ketamine anaesthesia (Imalgene, Rhône-Mérieux, France) and euthanized with sodium pentobarbital (SANOFI, Libourne, France). CSF samples were taken aseptically and stored at -80°C.

Isolation and culture of primary simian astrocytes

Astrocytes were prepared as previously described (Guillemin *et al*, 1997). Briefly, portions of cerebral cortex were extensively washed, minced finely and incubated with pure trypsin (0.25% w/v; Gibco-BRL, NY, USA) for 30 min at 37°C after disaggregation through a 10 ml pipette. After 20 min of incubation, 40 μl of DNase (Boehringer-Mannheim, Mannheim, Germany) at 10 u/ml was added for 10 min at 37°C. The suspension was centrifuged at $500 \times g$ for 5 min and the cells located at the top of the resulting pellets were resuspended in RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM glutamine (Boehringer-Mannheim, Mannheim, Germany), 1% PSN, 0.5% fungizone (Gibco, NY, USA), and 0.5% glucose. The cellular suspension was then cultured in 75 cm^2 tissue culture flasks (Falcon, USA). Cultures were kept for 3 days at 37°C in a 5% CO_2 containing water-saturated atmosphere. Adherent cells were grown to confluence for 10–15 days. At this time, 10 mM L-leucine-methyl-ester (LME) (Sigma, St Louis, USA) was added to the culture flasks for 1 h at room temperature to remove microglial cells and macrophages (Thiele *et al*, 1983). Then, cultures were rinsed twice with PBS at 37°C. After trypsinization (0.05% w/v), cells were cultured in RPMI 1640 medium supplemented with 10% FCS. The medium was changed twice a week after vigorous shaking.

The purity of the primary astrocyte cultures was systematically controlled by immunocytochemistry all along the culture. After 1 month of culture and treatment with LME, more than 99% of cells were positive for the astrocyte-specific marker GFAP. No cell was found positive for CD68, CD14, CD11b, CD4, NF160, Factor VIII, and galactocerebroside indicating that macrophages, microglia, neurons, endothelial cells and oligodendrocytes were absent or present below the level of detection (Guillemin *et al*, 1997).

In vitro *infection of simian primary astrocyte cultures*

SIVmac251 and SIVmac251-ΔNef (derived from the BK28 clone of SIVmac251 (Chakrabarti *et al*, 1995) and kindly provided by Dr AM Aubertin, Université Louis Pasteur, Strasbourg, France) were semipurified from culture supernatants of *in vitro* infected human cord blood mononuclear cells by ultracentrifugation in a SW41 rotor (40 000 r.p.m. for 45 min; Beckman), resuspended in RPMI medium and adjusted to 5 μg of p27/ml. Four to 6-week-old primary astrocyte cultures were trypsinised and 2.5×10^3 cells were plated into 10 cm^2 Flaskettes® (NUNC) in 2.5 ml of culture medium. After 48 h, the medium was replaced and supplemented with 2 μg/ml of polybrene (Sigma, St Louis, USA) and 10^7 u of anti-α-interferon (Sigma, St Louis, USA).

Before infection, viruses were incubated for 30 min at room temperature with 200 u/ml DNAse (Sigma Chemical Co, St Louis, MO). Cells were incubated for 24 h with 25 ng of p27/ml of SIVmac251 or SIVmac251-ΔNef (corresponding to approximately 50 000 c.p.m./ml of Reverse Transcriptase activity). As a negative control, astrocytes were infected with heat inactivated virus (1 h at 60°C). The cultures were then extensively washed with PBS and cultured as described above. Each infection experiment was performed three times with primary astrocyte cultures derived from different monkeys. Viral expression in cell free supernatants collected during the culture period was monitored by measuring Reverse Transcriptase (RT) activity (Retrosys, RT kit, Innovagen, Sweden), p27 (Coulter) and viral RNA (Chiron).

Treatment of astrocyte cultures with TNF-α or GM-CSF or IFN-γ

The medium of astrocyte cultures was removed and replaced with 2.5 ml of RPMI supplemented with 0.1% CSF and 100 iu/ml of either rh-TNF-α, rh-GM-CSF or rh-IFN-γ (Boehringer-Mannheim, Germany). Cytokine unit definitions were described by the

manufacturer. After 24 h supernatants were collected, cultures were washed with PBS, and 2.5 ml of RPMI medium supplemented with 10% FCS was added.

Antisera
Numerous antibodies that were used in immunocytochemical studies have been previously described (Guillemin *et al*, 1997). Rabbit antiserum to glial fibrillary acid protein (GFAP) was obtained from Sigma (St Louis, USA). The mAbs directed against SIVmac Nef (clones 2.2, 4.2, 9.2, 14.1 and 17.2), SIVmac Vpx (clone 1.2), and SIVmac Env SUgp120/160 (clones KK13 and KK18) came from Dr Kent, Dr K Krohn, and the MRC AIDS Reagent Project (NIBSC, Herts, UK). Monoclonal antibodies against SIVmac Rev (clones 2.1, 3.1, 4.1, 6.1 and 8.1) were provided by Dr V Ovod (University of Tampere, IMT, Finland), and mAbs against SIVmac Rev (clones 2.2, 3.1, 5.1 and 8.2) by Dr Kai Krohn and the MRC. Goat anti-mouse IgG1 and anti-rabbit fluorescein or Texas Red-conjugated antibodies were purchased from Southern Biotechnology Associated (Birmingham, USA), and used at the concentrations recommended by each manufacturer.

Immunocytochemistry
Cells were grown in permanox chamber-slides (Slide Flasks system; NUNC, Cergy Pontoise, France) for 2–3 days, and then fixed with 4% para-formaldehyde (PFA) for 30 min at room temperature. Afterwards, membranous permeablization was performed by incubation with 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were then rinsed three times with PBS and incubated with 5% normal goat serum (NGS) in PBS for 30 min at room temperature, rinsed twice with PBS and incubated for 1 h at 37°C with one or two (dual labelling) primary antibodies diluted in 5% NGS. The cells were then extensively washed with 5% NGS solution and incubated for 1 h at 37°C in the dark with the appropriate labelled secondary antibodies. After washing, cellular nuclei were stained using DAPI dyes (10 mg/ml). After washing several times with PBS at 37°C, the coverslips were mounted onto glass slides with Fluoromount-G® (SBA, Birmingham, USA), and examined with a Nikon fluorescence microscope.

p27 detection assay
The SIVmac p27 protein was quantified in supernatants from astrocyte cultures using the Coulter SIV core assay (Retrovirology Coulter Corporation, Hialeah, FL, USA). The baseline of significance for these experiments was fixed at 20 pg/ml using a large number of p27 culture supernatants and the recommendations of the manufacturer.

Quantification of viral RNA in astrocytes supernatants (Chiron)
SIV RNA levels in culture supernatants were evaluated using the branched-DNA technique (Bayer, Amsterdam, The Netherlands). The cut-off of this assay was 40 000 viral RNA copies per ml of supernatant.

Proviral DNA detection by Polymerase Chain Reaction
DNA samples were obtained using a rapid extraction method (Dynal, Compiegne, France). Two nested sets of oligonucleotide primers specific for the *env* and *pol* genes of SIVmac251 (Wakrim *et al*, 1996), and one nested set of oligonucleotide primers specific for the *env* gene of HIV-2 (Bayon-Auboyer *et al*, 1996) were used (Table 2). In our experimental conditions, the specific primer pairs did not allow cross-reaction between the SIVmac251 and HIV-

Table 2

Genes	*PCR*	*Primers*	*Sequences*	*Positions*
env	direct	LW9	5′-GAGAATACAGTCACAGAACAGG-3′	nucleotides 6814 to 6835
SIVmac251		LW10	5′-CATTGATTGGTTGTGAGTGG-3′	nucleotides 7575 to 7594
	nested	LW11	5′-CAACAATAACAACAGCAGCACC-3′	nucleotides 6962 to 6983
		LW12	5′-CCAAGTAGAAGTCTGTGTCTCC-3′	nucleotides 7386 to 7407
pol	direct	LW13	5′-ATTCCACTTACCAGTTGAGAGG-3′	nucleotides 3988 to 4009
SIVmac251		LW14	5′-CAGGTGTCTACTATCTGTCTGG-3′	nucleotides 4602 to 4623
	nested	LW15	5′-ACAGTGGTGGACAGACTATTGG-3′	nucleotides 4021 to 4042
		LW16	5′-GCCTGCTCTCTGATTCTGTAGG-3′	nucleotides 4352 to 4373
env	direct	6924an	5′-TGGCTTTAATGGCACTAGGGCA-3′	nucleotides 6924 to 6945
HIV-2		7523ac	5′-TCTGCCACCTCTGCACTAAAGG-3′	nucleotides 7544 to 7523
	nested	6952an	5′-AGAACATATATCTATTGGCATGGT-3′	nucleotides 6952 to 6975
		7276ac	5′-TCTCCTCTGCAGTTAGTCCACAT-3′	nucleotides 7298 to 7276
env	direct	BO4163an	5′-TCAGCTGCTTATCGCCATCTTGC-3′	nucleotides 6597 to 6619
SIVmac251		BO4164ac	5′-AGAACCTGCCGTTGCGAAAACC-3′	nucleotides 8185 to 8208
for	nested	BO4165an	5′-GTATGGCAACTCTTTGAGACCTCAATA-3′	nucleotides 6847 to 6873
sequencing		BO4161ac(bio)	5′-AGGCAAATAAACATTTTTGCCTAC-3′	nucleotides 7915 to 7938
	sequencing	BO4162an	5′-TGGCTTTAATGGAACTAGAGCA-3′	nucleotides 7410 to 7431
		BO4159ac	5′-TCTCCTCTGCAATTTGTCCACAT-3′	nucleotides 7756 to 7778

2ROD proviral sequences. Briefly, 1 μg of DNA was added to 100 μl of a preparation consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 100 μM dNTPs, 1.25 units of *Taq* polymerase (Appligene, Illkirch, France), and 0.25 μM of each oligonucleotide primer as described in Table 2. One precycle was run at 94°C for 3 min and 35 main cycles were run at 94°C for 45 s, at the optimal annealing temperature depending on each primer pair for 1.5 min and at 72°C for 1 min in a Crocodile DNA thermal cycler (Appligene, Illkirch, France). Positive and negative DNA controls were also run in the PCR reaction. Three microliters of the first PCR reaction was added to 97 μl of a second PCR mixture containing nested primers and amplified under the conditions stated above. Ten microliter aliquots of the PCR products were analysed by 1.5% agarose gel electrophoresis and ethidium bromide staining.

RT–PCR detection of SIVmac251 and HIV-2 mRNA in CSF

cDNAs were synthesised for 1 h at 42°C from 1 μg of RNA extracted from 100 μl of CSF using the guanidium thiocyanate method (Chomczyski and Sacchi, 1987), in a 40 μl final volume of 10 mM Tris-HCl (pH 8.3), 25 mM KCl, 0.6 mM $MgCl_2$, 1 mM dNTPs, 5 u/ml of Moloney Murine Leukaemia Virus reverse transcriptase (Gibco, NY, USA), 0.01 μg/ml of oligo$(dT)_{12-18}$, and 2 u/ml of recombinant RNase inhibitor (CloneTech Laboratories, Palo Alto, USA). PCR was performed on 5 μl of cDNA in a 50 μl reaction mixture following the same conditions as described above.

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