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Human immunodeficiency virus type 1 envelope-mediated neuropathogenesis: targeted gene delivery by a Sindbis virus expression vector

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Abstract

Sindbis virus (SIN) expression vectors offer the opportunity for studying neuropathogenesis because of their distinct neural cell tropism. Here, we demonstrate that a recombinant SIN vector expressing EGFP (SINrep5-EGFP) infected multiple cell types including neural cells from several species relevant to lentivirus pathogenesis with high levels of transgene expression. Infection of human neurons by a recombinant SIN (SINrep5-JRFL) expressing the full-length envelope from a neurovirulent human immunodeficiency virus type 1 (HIV-1) strain (JRFL) caused increased cytotoxicity compared to infection with SINrep5-EGFP ($P < 0.001$), while no cytotoxicity was observed among infected human astrocytes or monocytoid cells. Both human monocyte-derived macrophages (MDM) ($P < 0.01$) and astrocytes ($P < 0.001$) infected with SINrep5-JRFL released soluble neurotoxins in contrast to SINrep5-EGFP or mock-infected cells, although this was most prominent for the astrocytes. Implantation of SINrep5-JRFL into the brains of SCID/NOD mice induced neuroinflammation, neuronal loss, and neurobehavioral changes characteristic of HIV-1 infection, which were not present in SINrep5-EGFP or mock-infected animals. Thus SIN expression vectors represent novel tools for studying in vitro and in vivo HIV-1 neuropathogenesis because of their high levels of transgene expression in specific cell types within the brain.
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Introduction

Sindbis virus (SIN) is a prototypic member of the alphavirus family with a positive-strand RNA genome containing two open reading frames (ORF). The 5′ ORF encodes the viral replicase (the nonstructural proteins), which catalyzes genome replication and transcription, while the 3′ ORF encodes the structural proteins necessary for virus particle formation, which are translated from a subgenomic (sg) mRNA (reviewed in Strauss and Strauss, 1994). SIN together with the alphaviruses Semliki Forest virus (SFV) and Venezuelan equine encephalitis (VEE) virus have received increasing attention for their use as vectors for heterologous gene expression in molecular and cell biology studies (Schlesinger, 2000). More recently, there has been increasing interest in alphavirus vectors for use as therapeutic agents, especially for developing vaccines (Gardner et al., 2000; Schlesinger and Dubensky, 1999). Alphavirus vectors are appealing because of their wide host range and biological safety in humans (Strauss and Strauss, 1994). In many alphavirus vector systems, the structural proteins genes are replaced by a transgene of interest (Frolov et al., 1996; Schlesinger, 2000), permitting the viral replicase to replicate the genomic vector RNA and express the heterologous gene via the sg mRNA in the transfected cell. The self-replicating alphavirus vector containing a transgene of in-

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terest is packaged into a virus particle by addition of a defective helper virus construct, which supplies the requisite structural proteins (Frolov et al., 1996; Schlesinger, 2000).

The neurotropism of SIN is an attractive property for studying different aspects of central nervous system (CNS) function and disease. SIN vectors have been successfully exploited, expressing heterologous genes in brains of rats and mice (Altman-Hamamdzic et al., 1997; D'Apuzzo et al., 2001; Gwag et al., 1998), and are promising therapeutic tools (Lundstrom, 1999). SIN infection and replication may result in apoptotic death of the infected cell (Griffin and Hardwick, 1997). However, SIN vectors with reduced cytotoxicity and reduced replicative capacity in vitro have been developed and the extent of their cytotoxic effects depends on the cell type infected (Agapov et al., 1998; Dryga et al., 1997; Frolov et al., 1999). Limited data are available regarding the cytopathogenic effects of SIN vectors on neuronal cells in vitro and in vivo in the CNS. In this article we investigated the cytotoxicity of a SIN expression vector in vitro and in vivo and its potential for studying human immunodeficiency virus type 1 (HIV-1) neuropathogenesis.

Approximately 20% of untreated AIDS-defined patients will develop HIV-associated dementia (HAD) (McArthur et al., 1993), but the mechanisms underlying this neurological disorder remain uncertain. Infection by HIV-1 causes inflammation within the brain and neuronal degeneration, resulting in HAD (Power and Johnson, 2001). Both viral and host gene expression have been implicated in the pathogenesis of HAD (Chen et al., 1997; Corder et al., 1998; Power et al., 1998; Quasney et al., 2001; van Rij et al., 1999). Infection of the brain by HIV-1 and subsequent production of viral proteins in the brain are considered to play an important role in the neuropathogenesis of HIV-1 (reviewed in Kaul et al., 2001; Mollace et al., 2001; Power and Johnson, 2001). The HIV-1 envelope and Tat proteins are known to initiate several different cell-signaling pathways that lead to the production of different proinflammatory molecules. These molecules trigger other pathways that elicit the production of cytokines, matrix metalloproteinases, free radicals, and other unknown molecules with putative neurotoxic actions, ultimately resulting in neuronal degeneration or death. A SIN expression vector containing an HIV-1 gene offers the possibility of studying HIV-1 neuropathogenesis in a manner that resembles an infection, independent of other HIV-1 genes in vitro and in vivo as well as focusing on specific cell types and relevant regions of the brain. In addition, it allows the study of HIV-1 neuropathogenesis in hosts normally not susceptible to HIV-1 infection, such as mice and rats (Hofmann et al., 1999; Keppler et al., 2001; Lewis and Johnson, 1995; Mariani et al., 2000; Morrow et al., 1987; Wei et al., 1998). Herein we demonstrate that a recombinant SIN vector was able to infect several neural cell types relevant to HIV-1 neuropathogenesis, in both mouse and human primary cultures. Cytotoxicity of the SIN vector expressing enhanced green fluorescent protein (EGFP) was minimal in vitro and in vivo. Conversely, the same vector expressing the envelope protein of a brain-derived HIV-1 strain from a patient with HAD was neurotoxic in vitro and induced a neuroinflammatory response as well as neuronal injury in mice. Our findings indicate that SIN vectors can be used to express HIV-1 envelope protein in the brain and provide insight into the mechanisms underlying HIV-1 related neuropathogenesis.

Results

Tropism of SINrep5-EGFP in cell lines and primary cultures

Although alphavirus vectors have been shown to infect cells from different species in vitro (Lundstrom, 1999; Wahlfors et al., 2000), the extent of infection of cells from different species and lineages by SIN, especially those relevant to neuropathogenesis studies, required further investigation. To assess the ability of the SINrep5 vector system (Bredenbeek et al., 1993) to infect cells relevant for the study of HIV-1 neuropathogenesis, the ORF encoding the EGFP was inserted as a reporter gene into the SINrep5 vector, resulting in SINrep5-EGFP. Thus, the tropism of the recombinant SINrep5-EGFP virus in vitro was determined using immortalized cell lines and primary cell cultures (Table 1). As BHK-21 cells are highly susceptible to SIN infection, infection efficiency was determined relative to the number of infected BHK-21 cells using the same viral input titer and the same number of cells. SINrep5-EGFP infected neuronal and nonneuronal cell lines from mouse, rat, cat, and human, with differing infection efficiencies (Table 1). SINrep5-EGFP infected the human nonneuronal cell line 293T and the mouse neuroprogenitor cell line C17.2, with efficiencies of 48 and 22%, respectively. The Crandle feline kidney (CrFK) cells were infected with an efficiency of 7.6% relative to BHK-21 cells. All other cell lines derived from either mouse or human were infected with much lower efficiency compared to BHK-21 cells (1% or less). The human immortalized cell lines infected by SINrep5-EGFP included a cholinergic neuronal cell line (LAN-2), astrocytic cells (U373), and primary monocyte-derived macrophages (MDM) (Table 1, Fig. 1C). In all infected cells, the level of EGFP expression was high and easily detectable, which confirmed that the SINrep5-EGFP vector expressed efficiently in cells of different species, including humans.

As we were specifically interested in using SINrep5 for transgene expression in neural cells, the ability to infect cells in different primary CNS-derived cultures was determined with infection efficiency estimated relative to BHK-21 cells. SINrep5-EGFP readily infected neurons in primary cultures of fetal neurons from mouse, rat, and

Table 1
Tropism of SINrep5-EGFP for different cell lines and cell types

Cell type	Origin/description	Susceptibility to SINrep5-EGFP infection[a]
BHK-21	Baby hamster kidney	100%
C17.2	Mouse neuroprogenitor	22%
NG108	Mouse neuroblastoma	0.6%
CrFK	Crandle feline kidney	7.6%
Hela	Human cervical carcinoma	1.1%
293T	Human embryonic kidney epithilia	48%
U373	Human astrocytes	0.6%
Lan-2	Human cholinergic neurons	0.6%
Macrophages	Human monocyte derived macrophages	0.25%
Fetal neural stem cells	Mouse, primary culture	+
Adult neural stem cells	Mouse, primary culture	0.81%
Fetal neurons	Human, primary culture	+++
Embryonic striatal neurons	Rat, primary culture	++
Oligodendrocytes	Mouse, primary culture	−
Oligodendrocytes	Human, primary culture	−
Microglia	Human, primary culture	+
Astrocytes	Human, primary culture	+
Hippocampal slice	Rat, organotypic culture	++++

[a] Percentage of infected cells relative to number of infected BHK-21 cells using the same titer of SINrep5-EGFP. For primary cultures, − = no infection detected, + = 1–10%, ++ = 10–25%, +++ = 25–50%, ++++ = >50%.

human (Fig. 1 and Table 1), and infection of organotypic rat hippocampal tissue slices showed high levels of EGFP expression in pyramidal neurons in the CA1 and CA3 region (Fig. 1A). The results obtained with the organotypic hippocampal tissue slices corresponded with previous reports (Ehrengruber et al., 1999), although we observed that by adding virus to the media instead of direct injection into the tissue, selective infection of neurons occurred without glial infection (Fig. 1A). The interest in clinical and experimental applications of neural stem cells (Bjorklund and Lindvall, 2000) prompted us to assess their susceptibility to SIN infection. As shown in Fig. 1B and Table 1, both fetal and adult neural stem cells could be infected with SINrep5-EGFP and high levels of EGFP expression were observed. In addition, formation of cellular processes by the stem cells was also observed (Fig. 1B). In all primary cultures and rat hippocampal tissue slices, high levels of EGFP expression were observed without any obvious changes in cell morphology or loss of cells, 3–5 days after infection. Mixed cultures of human astrocytes and microglia were also successfully infected by SINrep5-EGFP, which was confirmed by immunostaining with antibodies directed against GFAP and CD14 (markers for astrocytes and microglia, respectively) (Fig. 1E–F). In contrast mouse and human oligodendrocytes in mixed cultures could not be infected by SINrep5-EGFP, which was confirmed by immunostaining with the H8H9 antibody directed against myelin basic protein (MBP) (data not shown). These results reflected the reported tropism of SIN in mice (Strauss and Strauss, 1994), but also extended earlier findings by demonstrating that SIN can also infect primary cells derived from the human CNS.

Infectivity and expression of HIV-1 envelope by SINrep5-JRFL

To explore the possibility of using a SIN expression vector as a tool to study HIV-1 neuropathogenesis, SINrep5-JRFL expressing the envelope protein of HIV-1 strain JRFL was constructed. JRFL is an HIV-1 strain derived from the brain of a patient with HAD (Li et al., 1991), and the envelope has potent neurotoxic actions (Power et al., 1998). Fig. 2A shows the expression of the HIV-1 JRFL envelope in the neural cell line NG108 infected with SINrep5-JRFL. Similar results were obtained for U373 cells infected with SINrep5-JRFL, and also in BHK-21, LAN-2, and 293T cells, in which JRFL envelope expression was readily detectable (data not shown). In addition, JRFL envelope is correctly processed and the surface unit (SU) gp120 released into the culture media of cells infected or transfected with SINrep5-JRFL, as illustrated by immunodetection of HIV-1 gp120 in culture media from BHK cells transfected with SINrep5-JRFL (Fig. 2B).

Cytotoxicity of SINrep5-EGFP and SINrep5-JRFL in vitro

In addition to susceptibility, relative EGFP expression levels were determined in the U373 (astrocytic), NG108 (neuronal), and BHK-21 cell lines, following infection using matched multiplicities of infection (m.o.i.) at which equal numbers of cells were infected for each cell line. The relative amounts of EGFP fluorescence measured (Fig. 3A), revealed that the U373 and NG108 cells infected with SINrep5-EGFP expressed significantly more EGFP compared to the infected BHK-21 cells ($P < 0.05$). This indicated that SINrep5 transgene expression was greater in the cells of neural origin using matched m.o.i.

To examine the intrinsic cytotoxicity of the SINrep5 vector in vitro, NG108, LAN-2, and U373 cells were infected with SINrep5-EGFP, because they closely resemble the cells infected or affected by in vivo HIV-1 infection and are infected with equal efficiency by the recombinant SIN vector. The levels of cell death among each cell line induced by infection with SINrep5-EGFP were minimal and similar to controls (Fig. 3B–D). To investigate the effect of expression of the HIV-1 JRFL envelope protein by SINrep5 on cell death in vitro, the cytotoxicity of SINrep5-JRFL in NG108, U373, and LAN-2 cells was also determined (Fig. 3B–D). The percentage of cell death after 24 or 48 h of infection revealed no significant difference in cytotoxicity of SINrep5-JRFL compared with mock or SINrep5-EGFP infections for the U373 cells. However, the cytotoxicity of the SINrep5-JRFL virus was significantly higher for NG108

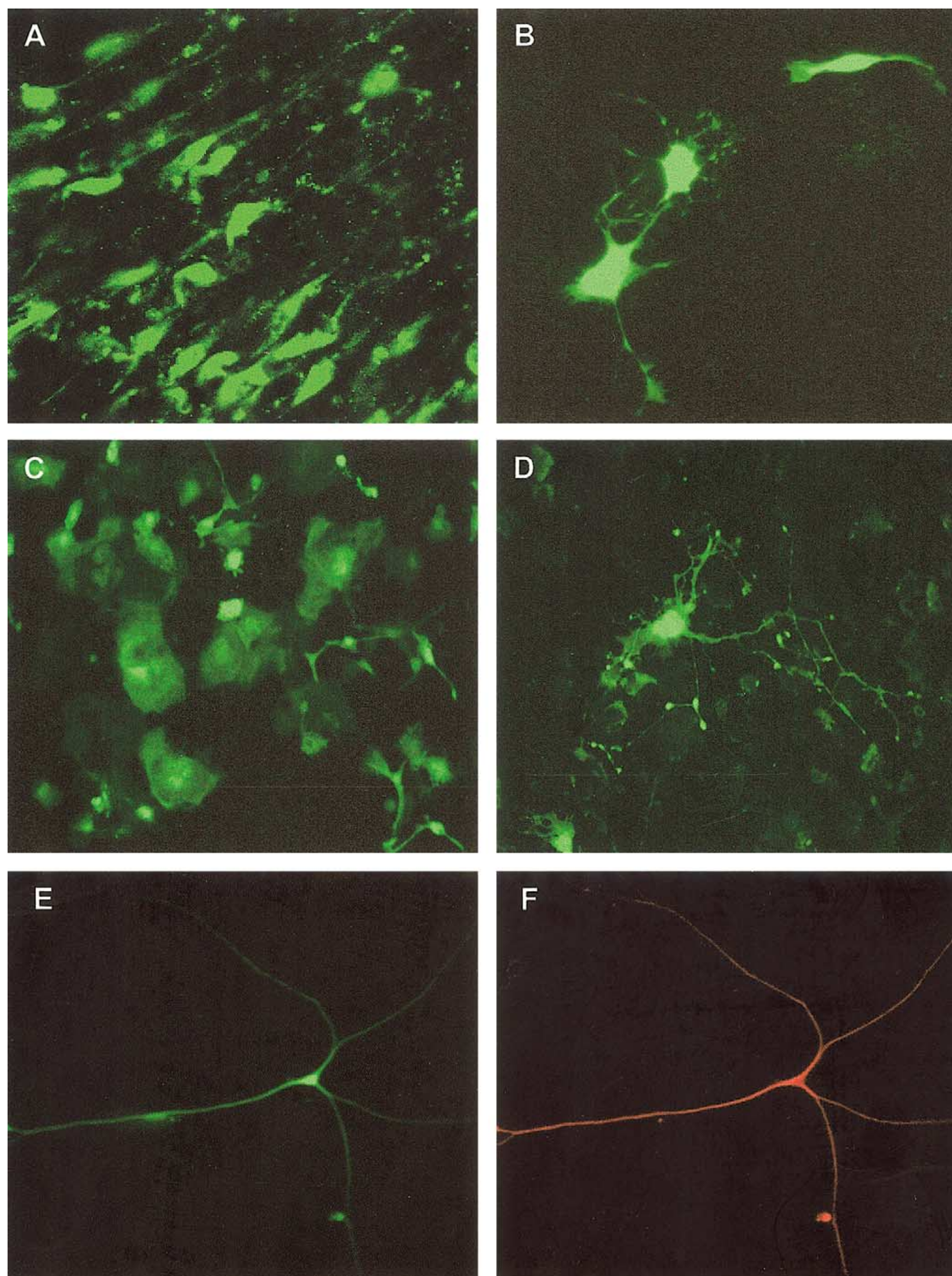

Fig. 1. EGFP expression in cells and rat hippocampal tissue slices infected with SINrep5-EGFP. (A) High levels of EGFP expression could be detected in pyramidal neurons of the CA1 region of hippocampal slices. (B) Mouse adult neuronal stem cells infected with SINrep5-EGFP expressed high levels of EGFP and were able to form cellular processes. SINrep5-EGFP also infected human MDM (C), primary human fetal neurons (D), and primary human astrocytes (E and F). The latter was determined by detection of EGFP fluorescence (E) in combination with immunostaining for the astrocyte marker GFAP (F), in mixed primary astrocyte cultures. (Original magnification 400×).

cells than for mock infection ($P < 0.05$) and increased after 48 h. Although mock infection for 48 h increased the percentage of dead cells compared to regular culture media, a significantly higher percentage of cell death was observed for infection of the human neuronal cell line LAN-2 with SINrep5-JRFL compared to SINrep5-EGFP and mock infection after 24 and 48 h ($P < 0.001$) (Fig. 3D). These results indicate that HIV-1 JRFL envelope expressed by SIN was cytotoxic in neuronal but not astrocytic cell lines.

Infection by HIV-1 results in activation of macrophages and astrocytes resulting in the release of soluble molecules with neurotoxic actions (Gonzalez-Scarano and Baltuch, 1999; Kaul et al., 2001). Similar to other lentiviruses including SIV and FIV, the release of neurotoxic molecules

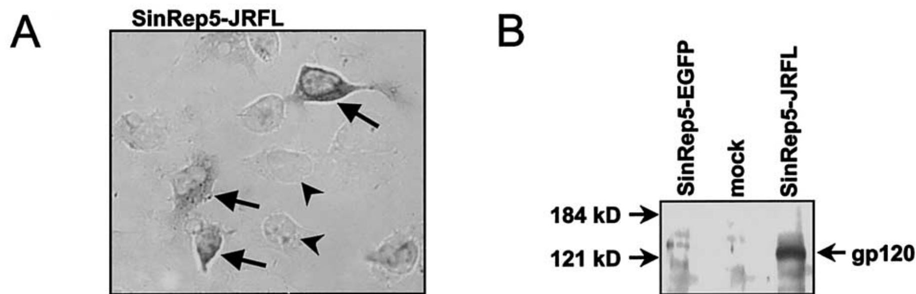

Fig. 2. HIV-1 envelop expression by SINrep5-JRFL. (A) NG108 cells infected with SINrep5-JRFL. HIV-1 envelope expressing cells are indicated with arrows, while immunonegative cells are indicated with arrowheads. (B) Western blot analysis of immunoprecipitation of the HIV-1 strain envelope protein from culture media from BHK cells transfected with SINrep5-JRFL, SINrep5-EGFP, and mock-transfected cells (mock), illustrating that the envelope expressed by SIN is correctly processed and the surface unit gp120 is released into the culture media.

by HIV-1 is in part mediated by the envelope protein (Johnston et al., 2002; Kaul and Lipton, 1999; Power et al., 1998). To determine if infection of macrophages and astrocytes by SINrep5-JRFL resulted in the release of soluble neurotoxins, human primary MDM and astrocytes were mock infected or infected with each SINrep5 vector. Infection of both MDM and astrocytes with SINrep5-JRFL or SINrep5-EGFP did not cause an increase in cell death for both cell types as determined by trypan blue exclusion after 24 h (data not shown). Incubation of LAN-2 cells for 24 and 48 h with conditioned media from MDM infected with SINrep5-JRFL resulted in an increase ($P < 0.01$) in neuronal death compared to incubation with conditioned media derived from SINrep5-EGFP and mock-infected MDMs (Fig. 4A). Although the cytotoxicity of conditioned media derived from MDM was limited compared to direct infection of LAN-2 cells with SINrep5-JRFL, these results indicated that SINrep5-JRFL also was able to induce indirect neurotoxic effects, similar to earlier reports of HIV-1 infection of MDMs (Power et al., 1998). More striking were the observations using the conditioned media from the human primary astrocytes (Fig. 4B) infected by SINrep5-JRFL, which induced extensive neuronal death that was not observed for SINrep5-EGFP or mock-infected astrocytes ($P < 0.001$). Indeed, incubation for 48 h with the conditioned media from SINrep5-JRFL-infected astrocytes resulted in 100% cell death, which again was not observed for the mock- or SINrep5-EGFP-infected astrocyte-derived conditioned media. Analysis of the conditioned media derived from the astrocytes and MDM by Western blot using the B13 antibody revealed that no detectable HIV-1 JRFL envelope was released from infected cells (Fig. 4C), in contrast to BHK cells infected by the same virus (Fig. 2B).

As stimulated astrocytes can release glutamate and glutamate receptors have been postulated to play a role in HIV-1-induced neuronal death (Bezzi et al., 2001; Kaul et al., 2001), the neurotoxicity of conditioned media from astrocytes was assessed in the presence of different concentrations of *N*-methyl-D-aspartate (NMDA; MK801) and amino-3-hydroxy-5-methyl-4-isoazole propionate (AMPA; NBQX) receptor antagonist (Fig. 4D). No reduction in neurotoxicity of conditioned media from SINrep5-JRFL-infected astrocytes was observed, indicating that the neurotoxins produced by HIV-1 envelope in astrocytes did not mediate neuronal death through the glutamate receptors.

Neurovirulence of SINrep5-EGFP and SINrep5-JRFL in vivo

Since a robust difference in cytotoxicity was observed between SINrep5-EGFP and SINrep5-JRFL in neuronal cell lines NG108 and LAN-2, the effects of both recombinant viruses were determined in vivo by stereotaxic implantation into the striatum of SCID/NOD mice. This brain region was selected because it is highly vulnerable to the effects of HIV infection (Glass et al., 1995). High levels of EGFP expression could be detected in brain tissue primarily along the needle track leading into the striatum by confocal fluorescent microscopy, at 1, 4, and 7 days after implantation with SINrep5-EGFP (Fig. 5B and C), but not in control animals (Fig. 5A). EGFP expressing could be easily detected for up to 10 days after implantation, which corresponds to previous reports (Altman-Hamamdzic et al., 1997). The cells infected by SINrep5-EGFP after striatal implantation were primarily neurons. Immunostaining of brain sections with an antibody directed against EGFP confirmed this observation (data not shown). Following infection with SINrep5-JRFL, HIV-1 envelope could be detected at the injection site at 7 days after infection using a monoclonal antibody directed against HIV-1 envelope (Fig. 5D). To determine the identity of the HIV-1 envelope-positive cells, tissue sections were double labeled using the antibodies against ionized calcium-binding adaptor molecule 1 (Iba-1) or glial fibrillary acidic protein (GFAP) and the HIV-1 envelope antibody followed by confocal microscopy (Fig. 5E and F). This revealed that the HIV-1 envelope immunopositive cells surrounding the injection site were Iba-1 positive, indicating they were of microglial lineage. No colocalization of GFAP and HIV-1 envelope immunoreactivity was observed. In addition, the rotational behavior of the mice was also analyzed 3 and 7 days after striatal implantation to determine the cytotoxic effects of the virus and delivery method (Un-

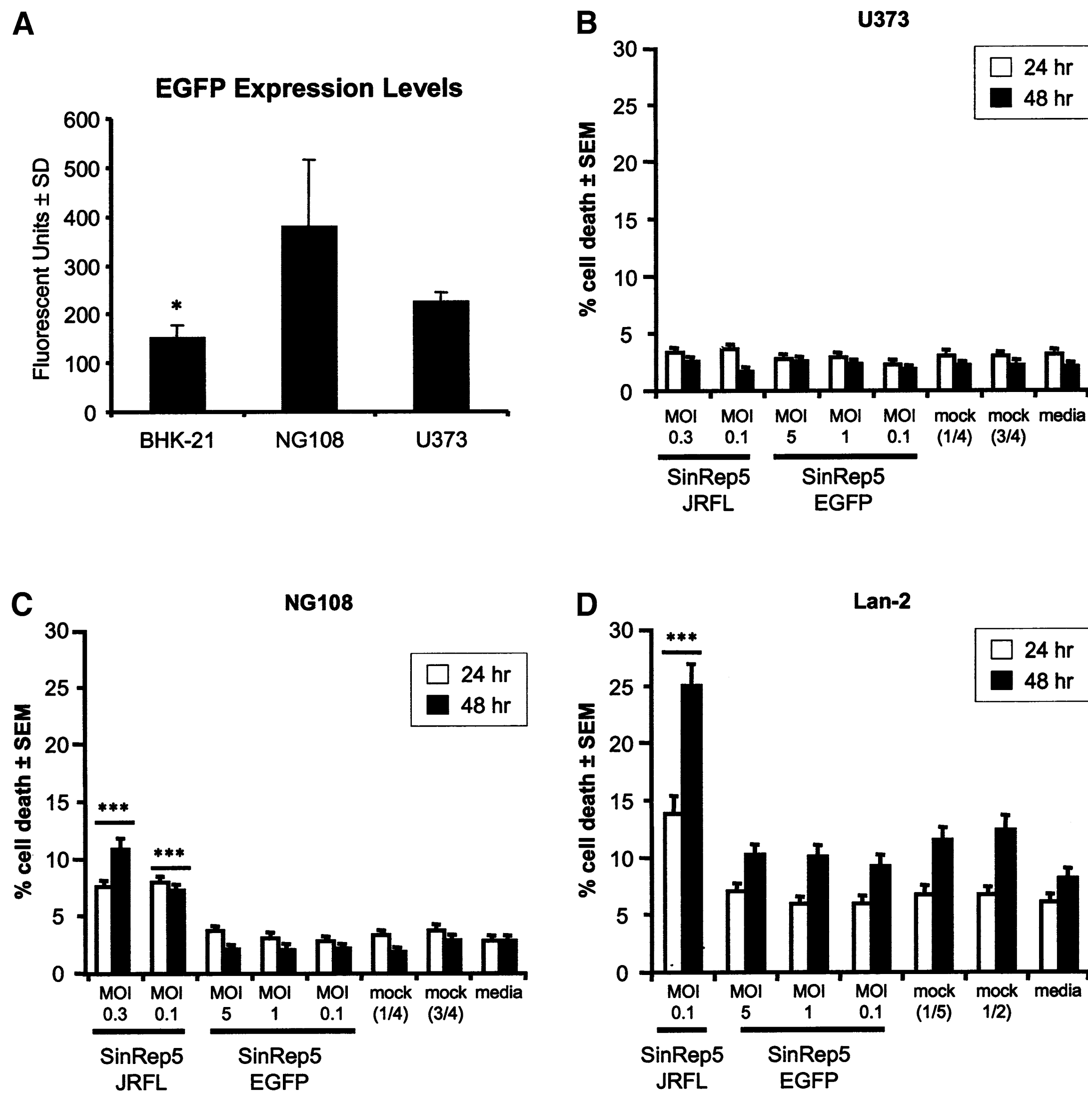

Fig. 3. Expression levels of SINrep5 in BHK-21, NG108, and U373 cells as determined by measuring EGFP fluorescence (A). NG108 and U373 cells displayed significantly more EGFP fluorescence (i.e., EGFP expression) compared to BHK-21 cells. (* $P < 0.05$ Dunn's multiple comparison test). Induction of cell death on U373 (B), NG108 (C), and LAN-2 cells (D) by SINrep5-EGFP and SINrep5-JRFL. Cells were infected at several M.O.I. for 24 and 48 h, and cell death was assessed by trypan blue exclusion 24 and 48 h after infection. Cell death induced by media from mock-transfected BHK cells (mock) mixed in various ratios with culture media (ranging from 3/4 to 1/4) as well as culture media alone on the different cell lines were used as controls. Cell death was significantly increased in the neuronal cell lines LAN-2 and NG108 infected with SINrep5-JRFL compared to uninfected (mock and AIM-V) and SINrep5-EGFP-infected cells (***$P < 0.001$, Dunn's multiple comparison test).

gerstedt and Arbuthnott, 1970). The total mean number of rotations (±SEM) of the animals implanted with SINrep5-JRFL was significantly lower ($P < 0.05$, Mann-Whitney U test) at day 7, but not day 3, compared to the SINrep5-EGFP-implanted animals (67 ± 5 and 119 ± 11 rotations/10 min, respectively), which did not differ from control animals, indicating that neurological injury was caused by implantation of SINrep5-JRFL but not SINrep5-EGFP.

To determine if this neurobehavioral deficit was correlated with neuropathological changes, analysis of frozen brain sections immunostained with antibodies directed against Iba-1 and GFAP revealed minimal activation of microglia and astrocytes, respectively, surrounding the injection site in control (Fig. 6A and D) and SINrep5-EGFP (Fig. 6B and E)-implanted animals. However, the number of both Iba-1 and GFAP immunopositive cells and the intensity of staining were markedly increased in the animals implanted with SINrep5-JRFL (Fig. 6C and F) compared to SINrep5-EGFP and control animals. Moreover, neuronal loss was also observed at the site of injection and away from the needle track in the animals implanted with SINrep5-JRFL after immunostaining for the neuronal nuclear protein (NeuN) (Fig. 6I) and cresyl violet staining (data not shown), which was not apparent in the control animals or animals implanted with SINrep5-EGFP (Fig. 6G and H). These findings suggested that the neuronal damage caused by

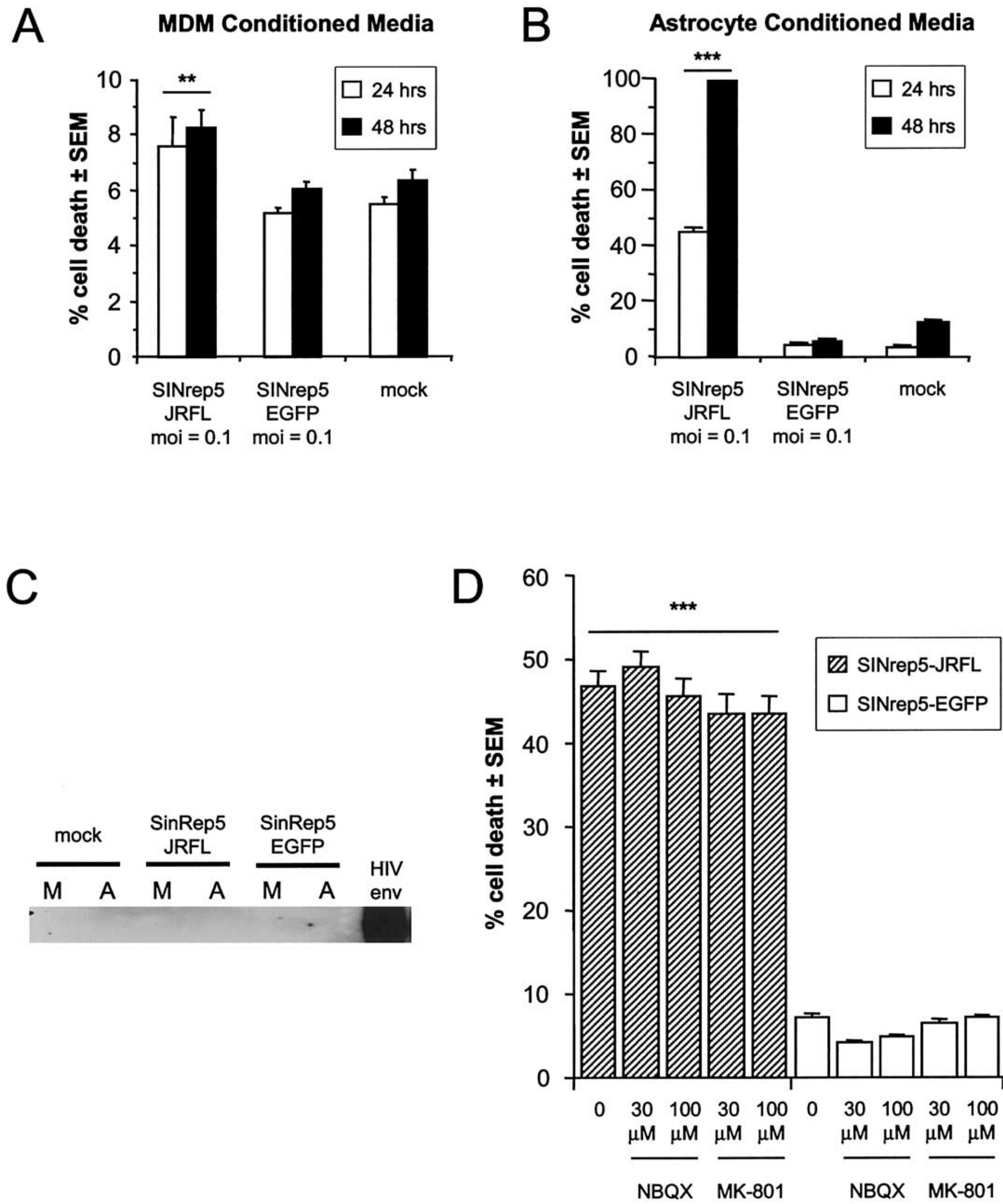

Fig. 4. Neurotoxicity in LAN-2 cells of conditioned media from human MDM (A) and primary human astrocytes (B) infected with SINrep5-EGFP, SINrep5-JRFL, or uninfected (mock). Cells were incubated for 24 and 48 h with conditioned media from astrocytes and cell death was assessed by Trypan blue exclusion. Cell death was significantly increased in LAN-2 cells upon incubation with conditioned media of primary human astrocytes infected with SINrep5-JRFL at both time points compared to mock and SINrep5-EGFP, while only a modest increase was observed for the MDM conditioned media (**$P < 0.01$, ***$P < 0.001$, Dunn's multiple comparison test). (C) Western blot analysis of media of mock-, SINrep5-EGFP-, and SINrep5-JRFP-infected astrocytes (A) and MDM (M). No detectable levels of HIV-1 JRFL envelope (gp120) were observed in both astrocyte- and MDM-conditioned media. HIV-1-infected 293T cells were used as positive control (HIV env gp120). (D) Neurotoxicity in LAN-2 cells after 24 h treatment with conditioned media from primary human astrocytes infected with SINrep5-EGFP and SINrep5-JRFL in the presence or absence of the AMPA (NBQX) and NMDA (MK801) receptor antagonists. Media from astrocytes infected with SINrep5-JRFL induced significantly more cell death compared to the background level of cell death induced by conditioned media of astrocytes infected with SINrep5-EGFP (***$P < 0.001$, Dunns multiple comparison test) and no reduction of cell death was observed in the presence of NBQX or MK801.

SINrep5-JRFL led to the neurobehavioral abnormalities observed in this group of animals. Overall, these results revealed that SINrep5-JRFL induced more neuropathogenic effects upon implantation into the striatum of mice compared to SINrep5-EGFP.

Discussion

In this report we demonstrate that a recombinant SIN is able to infect and efficiently express EGFP in human primary neural cells and cell lines relevant to HIV-1 patho-

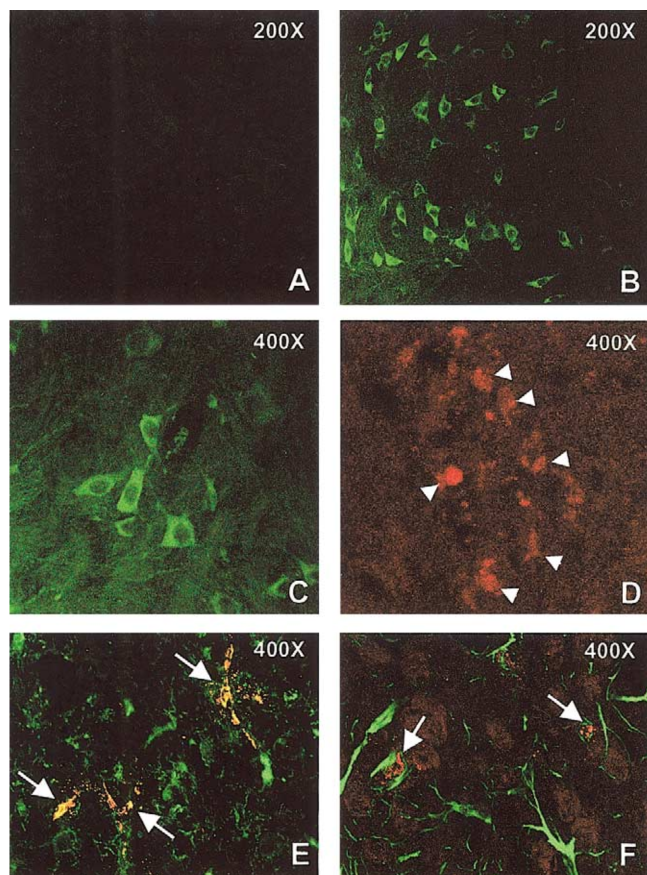

Fig. 5. In vivo detection of EGFP and HIV-1 envelope expression detected by confocal microscopy. EGFP was detected in neurons of the striatum of SCID/NOD mice 7 days after implantation of SINrep5-EGFP (B and C), while no fluorescence was observed in control animals (A). HIV-1 JRFL envelope could be detected (indicated with arrowheads) by immunofluorescence, 7 days after implantation of SINrep5-JRFL (D). Double immunolabeling for HIV-1 envelope (red) and the activated microglial marker Iba-1 (green) (E) and GFAP (green) (F) in SINrep-JRFL implanted, revealed colocalization (in yellow) of the envelope protein with the microglial marker Iba-1, but not with the astrocyte marker GFAP in the SINrep5-JRFL-implanted animals (HIV-1 envelope-positive cells indicated with arrow in E and F). (Original magnification as indicated).

genesis without apparent cytotoxicity. Conversely, infection by a recombinant SIN expressing the envelope derived from a neurovirulent HIV-1 strain resulted in increased neuronal death in vitro. Moreover, HIV-1 envelope expression in astrocytes induced significant indirect neurotoxicity. Implantation of this recombinant virus into the brain of mice induced a marked inflammatory response and neuronal loss, together with neurobehavioral abnormalities. These findings indicate that the HIV-1 envelope expressed by SIN vectors retains its neurotoxic properties both in vitro and in vivo, and illustrate the potential of SIN expression vectors for studying the neuropathogenesis of individual viral genes.

As it was essential to gain more insight into the cell tropism of SIN, we tested the ability of SINrep5-EGFP to infect neuronal and nonneuronal cells of mouse, rat, feline, and human origin. The present studies extended earlier studies (Lundstrom, 1999; Strauss and Strauss, 1994; Wahlfors et al., 2000) by showing that several primary neural cell types of different species could be infected. In addition, the ability of the SINrep5-EGFP virus to infect mouse fetal and adult neural stem cells suggests SIN vectors could also be exploited to express genes that have investigative and therapeutic value in stem cell studies (Bjorklund and Lindvall, 2000). Of special interest was the ability of SINrep5-EGFP to infect the human neuronal cell line LAN-2, the astrocytic cell line U373, and human MDM, which was complemented by the infection of human primary cultures showing a similar tropism for microglia (the brain resident cells from monocyte/macrophage lineage), astrocytes, and neurons. The later findings indicate that in vitro SIN infects the same cell types in primary human-derived neuronal cultures as in mice. Astrocytes and macrophages are considered to play a major role in the pathogenesis of many brain diseases including HAD (Kaul et al., 2001; Kaul and Lipton, 1999; Nath et al., 1999; Thompson et al., 2001). Cells of monocyte/macrophage lineage have been proposed to act as the vehicles by which HIV-1 may cross the blood brain barrier (BBB) and infect the brain (Strelow et al., 2001). Moreover, infection by HIV-1 of astrocytes and cells of macrophage/microglial lineage within the brain results in the release of soluble viral and host molecules with neurotoxic effects (Kaul et al., 2001; Mollace et al., 2001; Power and Johnson, 2001). Neurons in the brain are also infected by HIV-1, albeit at much lower levels (Bagasra et al., 1996; Nuovo et al., 1994; Torres-Munoz et al., 2001; Trillo-Pazos et al., 2002).

To explore the possibility of utilizing SIN vectors in the study of HIV-1 neuropathogenesis, SINrep5-JRFL was constructed because the HIV-1 envelope is considered to be a principal viral gene influencing neuronal injury and death in HAD (Kaul et al., 2001; Power and Johnson, 2001). Given that SINrep5-EGFP caused negligible cell death in all three cell lines even at m.o.i.'s 50 times higher than SINrep5-JRFL, the increased cell death in the neuronal cell lines NG108 and LAN-2 upon SINrep5-JRFL infection was the result of the expression of the JRFL envelope. In addition to its direct neurotoxic effects, SINrep5-JRFL also induced production of neurotoxic molecules by primary astrocytes and to a much lesser extent in MDM. The high level of neurotoxicity of the conditioned media from the primary astrocytes suggests that astrocytes may also be an important source of soluble neurotoxins induced by HIV-1 envelope expression. As no detectable HIV-1 gp120 was present in the conditioned media from SINrep5-JRFL-infected MDM and primary astrocytes, other molecules released as a response to expression of the HIV-1 envelope must be responsible for the increased cytotoxicity of the astrocyte-conditioned media. Moreover, the inability to block the neurotoxic effects of the conditioned media derived from the primary astrocytes infected by SINrep5-JRFL with antagonists for the NMDA and AMPA receptors indicated that these neurotoxins do not act via the glutamate receptors, in

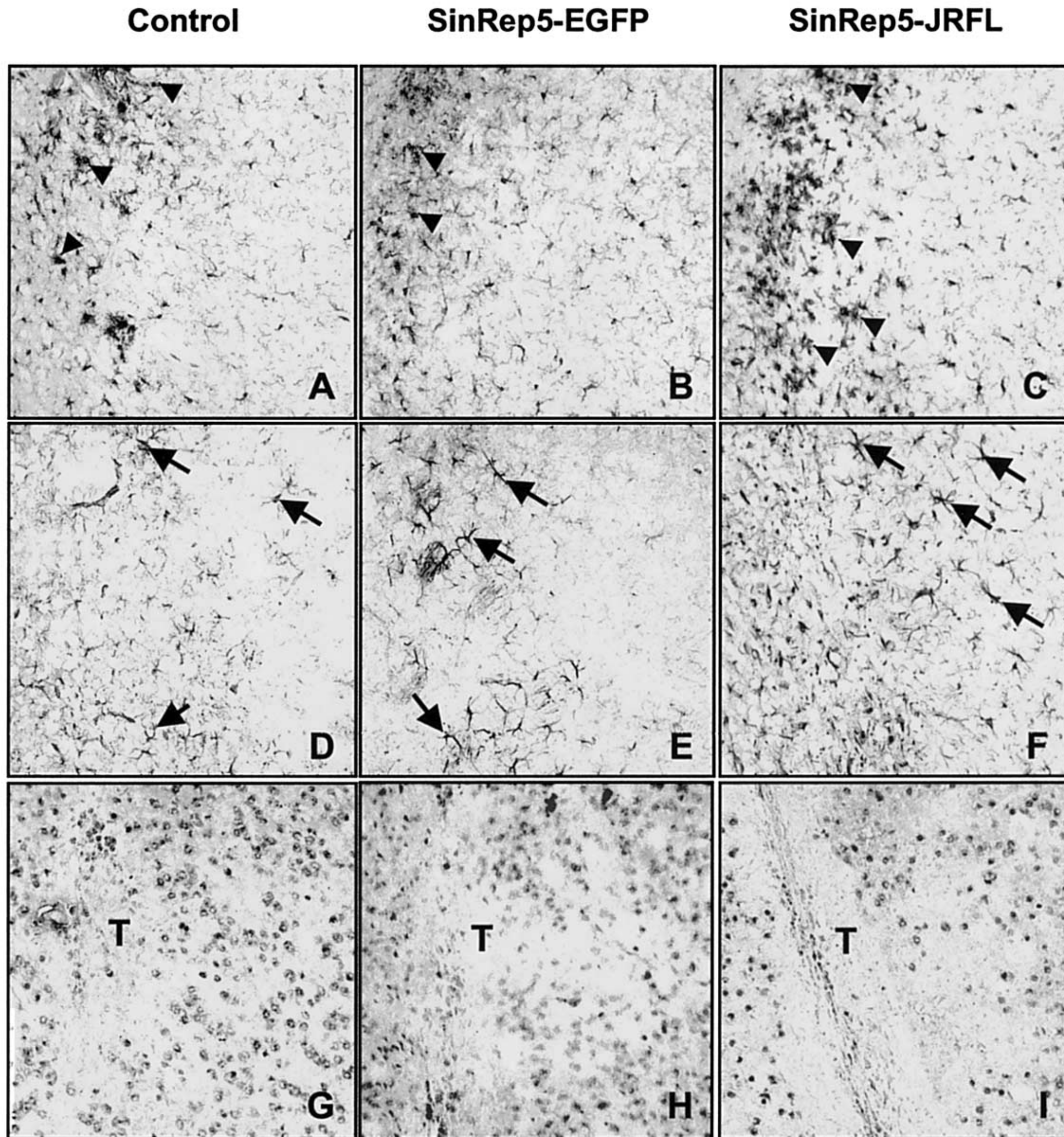

Fig. 6. Neuropathological findings in brain of SCID/NOD mice following control stereotactic implantation (A, D, and G) or implantation with SINrep5-EGFP (B, E, and H) and SINrep5-JRFL (C, F, and I). Expression of cell markers for inflammation and activation of microglia (Iba-1) (A, B, and C), astrocytes (GFAP) (D, E, and F), and neuronal nuclei marker (NeuN) (G, H, and I), in striatal sections 7 days after implantation. Immunopositive microglia are indicated with arrowheads and astrocytes with arrows. An increased inflammatory response with a loss in the number of neurons in the vicinity of the needletrack (T) and surrounding tissue was observed in animals implanted with SINrep5-JRFL that was not present in control animals or animals implanted with SINrep5-EGFP. (Original magnification 200×).

contrast to earlier studies of astrocyte-mediated neurotoxicity (Araque et al., 1998; Parpura et al., 1994). However, altered intracellular expression of viral envelope has been previously associated with neurovirulence (Kamps et al., 1991; Lynch et al., 1995; Lynch and Sharpe, 2000), possibly through misfolding of the protein in the endoplasmatic reticulum, which results in a stress response by the cell. The stress response may lead to the release of potentially neurotoxic molecules, including cytokines and reactive oxygen species (Wesselingh and Thompson, 2001). The mechanism underlying the astrocyte-mediated neurotoxicity is currently under further investigation.

The lack of T lymphocytes in SCID/NOD mice permits the use of these animals as model for HIV-1 neuropathogenesis by implanting recombinant HIV-1 proteins and human cells infected with HIV-1 or expressing specific HIV-1 proteins (Johnston et al., 2001; Persidsky et al., 1996; Tyor et al., 1993). The robust innate inflammatory response caused by SINrep5-JRFL consisting of microglial and astrocyte activation are hallmarks of HAD (Power and Johnson, 2001), indicating the SINrep5-JRFL-implanted SCID/NOD displayed the typical features of HIV-1 infection in terms of site of injury (the striatum) and pathological changes. The lower total rotary behavior observed for the

SINrep5-JRFL-implanted animals was indicative of motor abnormalities resulting from the neurotoxicity of the HIV-1 JRFL envelope. Moreover, the titer of SINrep5-JRFL implanted was 60 times lower than the SINrep5-EGFP input titer, underscoring the neuropathogenic features of the HIV-1 envelope. As our in vitro data showed that SINrep5-JRFL is toxic to neurons, these observations indicate that the infected neurons may have died, leaving only the SINrep5-JRFL-infected cells of microglial lineage 7 days after infection. Alternatively, the microglia and macrophages could have internalized the HIV-1 envelope during the process of phagocytosing the SINrep5-JRFL-infected neurons. The observed neuronal loss and the presence of HIV-1 envelope in microglia complements an in vitro study by Boutet et al. in which microglia infected with an HIV-1 envelope expressing SFV vector induced cell death in uninfected bystander cells, but not in the infected microglia (Boutet et al., 2000).

Many studies demonstrating neurotoxicity of HIV-1 envelope have been performed using purified recombinant envelope protein, frequently from a non-brain-derived HIV-1 strain (Bagetta et al., 1996; Bansal et al., 2000; Bennett et al., 1995; Corasaniti et al., 2001), or in transgenic animals expressing one or more HIV-1 proteins (Hanna et al., 1998a, b, 2001; Jolicoeur et al., 1992; Toggas et al., 1994). Given that we show that SIN infects human and murine cell types relevant to HIV-1 pathogenesis, the use of SIN vectors for expression of HIV-1 genes ex vivo and in vivo offers a novel tool for elucidating mechanisms of neuropathogenesis. Furthermore, the transient expression of heterologous genes by SIN vectors, the minimal intrinsic cytoxic properties of individual vectors, and the broad host cell tropism makes these vectors appealing for pathogenesis studies but also as therapeutic tools for acute neurological diseases.

Material and methods

Construction of SINrep5-EGFP and SINrep5-JRFL

The SIN vector system used in this study has been described previously (Bredenbeek et al., 1993). The constructs pSINrep5-EGFP and pSINrep5-JRFL were obtained by cloning the EGFP and HIV-1 envelope genes, respectively, into the multiple cloning site present in the pSINrep5 expression vector. To construct pSINrep5-EGFP, pEGFP-N1 (Clontech, Palo Alto, CA) was digested with *Sma*I and *Hpa*I, and the resulting 862-bp DNA fragment, containing the EGFP ORF, was isolated from an agarose gel using the Concert Rapid Gel extraction kit of Life Technologies Inc. (Burlington, ON) and inserted into the *Stu*I restriction site of the pSINrep5 multiple cloning site. SINrep5-JRFL was constructed by inserting the envelope ORF of the brain-derived HIV-1 strain JRFL (Li et al., 1991) into pSINrep5. The HIV-1 JRFL envelope insert was obtained by digesting the expression vector pCMV-JRFL (Zhang et al., 2001) with *Kpn*I and *Bam*HI and subsequent isolation from an agarose gel. The 2.5-kb fragment was inserted into the *Kpn*I and *Bam*HI restriction sites of the polylinker of pSL1180 (Amersham Pharmacia Biotech, Baie d'Urfe, QC). The resulting construct pSL-JRFLenv was digested with *Sma*I and *Hpa*I to generate a 2.5-kb fragment containing the HIV-1 JRFL envelope ORF, which was subsequently cloned into the *Stu*I site of the multiple cloning site of pSINrep5. For all constructs correct insertion of the inserts was determined by restriction enzyme digest analysis and sequencing. All restriction and other enzymes were obtained from New England Biolabs (Mississauga, ON) or Life Technologies and used according to their specifications.

Production of SINrep5-EGFP and SINrep5-JRFL virus stocks

Constructs pSINrep5-EGFP and pSINrep5-JRFL and helper virus construct pDH-BB (Bredenbeek et al., 1993) were linearized with *Xho*I. The linearized plasmids were used as templates for the generation of capped RNA transcripts by in vitro run-off transcription using the SP6 mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX). Ten micrograms of DH-BB RNA transcript and 10 μg SINrep5-EGFP or SINrep5-JRFL RNA transcript was transfected to 3–5 $\times 10^6$ BHK-21 cells/ml in PBS by electroporation according to a previously published protocol (Bredenbeek et al., 1993). Briefly, the cells were subjected to two pulses at 850 V, 50 μF, and $\infty\Omega$, using a Gene Pulser II (Bio-Rad Laboratories Canada Ltd., Missisauga, ON). Cells were plated in culture media and incubated at 37°C/5% CO_2. The media containing recombinant SIN was harvested 20–24 h after transfection. The SINrep5-JRFL viral stock was concentrated by centrifugation at 120,000 *g* at 4°C for 4 h and the virus pellet was dissolved in culture media. The recombinant SIN stocks were titered on BHK cells. For SINrep5-EGFP the titer was determined by counting the number of EGFP-positive cells using fluorescent microscopy 24 h postinfection. To determine the titer of SINrep5-JRFL, cells were immunostained using monoclonal antibody B13 directed against the HIV-1 envelope (Abacioglu et al., 1994) and the number of HIV envelope-positive cells were counted. On average 5×10^6 to 3×10^7 infectious virus particles per milliliter were obtained for SINrep5-EGFP and 4×10^4 particles/ml (0.5×10^6 particles/ml after concentration) were obtained for SINrep5-JRFL.

Infection and transfection of neuronal and nonneuronal cell lines

The SINrep5-EGFP virus stocks were used to infect neuronal and nonneuronal cell lines derived from different species including C17.2 (mouse neuroprogenitor) (Snyder et al., 1992), NG108 (mouse neuroblastoma), LAN-2 (human cholinergic neurons, a gift from Dr. J. Rylett, Univer-

sity of Western Ontario), and U373 (human transformed astrocytes), seeded typically at densities of between 2.5 and 5×10^4 cells/cm^2. The nonneuronal cell lines used were CrFK, HeLa (human cervical carcinoma), and 293T (human embryonic kidney epithelia). The HeLa, NG108, U373, CrFK, and 293T cell lines were all obtained from the ATCC. All cells were infected using serial dilutions of the virus stock. Percentage infection efficiency of the different cell lines was determined by counting the number of EGFP-positive cells relative to the number of EGFP-positive cells produced by the same amount of virus on BHK-21 cells. The different cell lines were also used to introduce the SINrep5-EGFP RNA by electroporation.

In addition, the level of EGFP transgene expression in BHK-21, NG108, and U373 cells was determined by infecting cells seeded in 96-well plates at 10^4 cells/well with SINrep5-EGFP at a matched m.o.i., resulting in equal numbers of infected cells (17 and 35%, respectively) and determining the amount of EGFP fluorescence in the cells 24 h postinfection using a fluorescent plate reader (Wallac VICTOR^2 1420 multilabel counter; Perkin–Elmer, Wellesley, MA). Infection of each cell line was repeated in triplicate in two separate experiments.

Infection of primary neuronal cultures and neural stem cells

Primary cultures were obtained for rat embryonic striatal neurons, mouse adult (postnatal day 21) oligodendrocytes, mouse fetal neurons, human fetal neurons, and mixed cultures of human adult oligodendrocytes, astrocytes, and microglia. All primary cultures were prepared according to previous described protocols (Corley et al., 2001; Vecil et al., 2000; Weiss et al., 1993) and were kindly provided by the laboratories of Drs V.W. Yong and S. Weiss, University of Calgary. To infect the primary cultures, 10^5 infectious particles of SINrep5-EGFP were added to culture media. The cultures were incubated at 37°C/5% CO_2 for up to 2 days. Infection was determined by fluorescent microscopy for EGFP using a standard fluorescent microscope setup (Axioskop 2, Carl Zeiss Canada Ltd., Toronto, ON). Primary cultures were also immunostained with antibodies directed against oligodendrocyte (MBP, Serotec, Oxford, UK), astrocyte (GFAP, Dako, Glostrup, Denmark), and microglial (CD14) markers to determine the identity of the infected cells and assess the cell composition of the primary cultures. Adult and embryonic neural stem cells were isolated from mice according to previously published protocols (Daadi and Weiss, 1999). Stem cells were infected for 1 h at 37°C/5% CO_2, and infection was determined by fluorescent microscopy for EGFP after 24 and 48 h.

Infection of organotypic hippocampal tissue slices

Organotypic rat hippocampal slices were prepared as previously described (Stoppini et al., 1991). Slices were in culture for 7 to 14 days before infection with SINrep5-EGFP. Hippocampal slices were infected by adding 10^5 infectious virus particles per milliliter to the culture media and by incubating for 1–5 days. The tissue slices were fixed in PBS/4% paraformaldehyde 1, 2, 3, and 5 days after infection and infection of the tissue slices was determined by confocal laser scanning microscopy (Armstrong and MacVicar, 2001) for EGFP.

Analysis of cytotoxicity of SINrep5-EGFP and SINrep5-JRFL in vitro

NG108, LAN-2, and U373 cells were infected for 24 and 48 h with SINrep5-EGFP or SINrep5-JRFL using an m.o.i. of 0.1, 1, and 5 for SINrep5-EGFP and 0.1 and 0.3 for SINrep5-JRFL. As control, equal volumes of conditioned medium, obtained from mock transfected BHK cells, were incubated with the different cell lines (mock). After 24 and 48 h the percentage of cell death was assessed by trypan blue exclusion and compared to the mock-infected controls. The cytotoxicity of both recombinant viruses was tested in triplicate in two separate experiments for each cell line. The indirect cytotoxic effects of the recombinant SIN virus vectors were tested by infecting human MDM, as well as human primary astrocytes, with SINrep5-EGFP and SINrep5-JRFL for 4 h at 37°C/5% CO_2. All MDM infections were repeated in triplicate using MDM derived from three different donors/individuals. The astrocyte infections were performed in duplicate. The inoculum was replaced with AIM-V media and cells were incubated for 20 h at 37°C/5% CO_2. In addition, cell death in human MDM and primary astrocytes was measured by Trypan blue exclusion 24 h after the initial infection by SINrep5-EGFP, SINrep5-JRFL, and mock infection. Subsequently, the conditioned media of the MDM and primary astrocytes were tested for neurotoxicity by incubating the human LAN-2 cell line for 24 and 48 h with that conditioned AIM-V media and by determining cell death by trypan blue exclusion. As control, conditioned media of MDM and primary astrocytes incubated with media from mock-transfected BHK cells were used. The cytotoxicity of conditioned media derived from the MDM and primary astrocytes was tested in triplicate in two separate experiments. To assess the role of glutamate receptors in neuronal death, the neurotoxicity of conditioned media derived from primary astrocytes was also determined in the presence of 30 and 100 μM of the NMDA and AMPA receptor antagonists NBQX and MK801, respectively (Sheardown et al., 1990; Wong et al., 1986) (Sigma-Aldrich Canada Ltd., Oakville ON).

Stereotactic implantation of SINrep5-EGFP and SINrep5-JRFL in the mouse striatum

The right striata of 3-week-old SCID/NOD mice were stereotacticaly implanted with SINrep5-EGFP (3×10^7

particles/ml) (n = 8) and SINrep5-JRFL (0.5 × 10^6 particles/ml) (n = 4), in a volume of 2 μl over 5 min in accordance with CACCA guidelines. Control mice were implanted with media from mock-transfected BHK-21 cells (n = 3) or PBS (n = 4). As no differences were observed in behavior and inflammatory responses in the brain between these two groups, these animals will be addressed as controls for ease of discussion. The coordinates for the implant were 3 mm posterior, 2.5 mm lateral, and 3 mm deep relative to the bregma. Rotary behavior of the implanted animals was analyzed at 3 and 7 days after implantation, following intraperitoneal injection of 1 mg/kg amphetamine (Johnston et al., 2001; Ungerstedt and Arbuthnott, 1970). Animals were sacrificed at day 1, 3, and 7 and intracardially perfused with saline followed by PBS/4% paraformaldehyde. Subsequently, the brain was removed and postfixed in PBS/4% paraformaldehyde until histochemical analysis.

Immunohistochemistry

Fixed brain tissue was incubated for 72 h in 30% sucrose in PBS, embedded, and frozen on dry ice in Tissue Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA). Sections of frozen tissue measuring 10 μm were cut using a cryostat (Leica, Richmond Hill, ON). The tissue slices were analyzed by direct fluorescent microscopy for EGFP using a confocal microscope (Fluoview FV300, Olympus, Markham, ON). An EGFP-specific antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was also used to visualize infected cells. A monoclonal antibody B13, directed against HIV-1 envelope (Abacioglu et al., 1994), was used to detect HIV-1 envelope expression in animals infected with SINrep5-JRFL. In addition, tissue sections were immunostained using antibodies directed against GFAP, Iba-1 (Imai et al., 1996; Ito et al., 1998), and neuronal nuclei (NeuN) (Chemicon Int., Temecula, CA) using previously described procedures (Power et al., 1993).

Statistical analyses

All statistical analyses were performed using Graphpad InStat Version 3.01 (GraphPad Software, San Diego, CA) and P values of less than 0.05 were considered significant.

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