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Replication of different clones of human immunodeficiency virus type 1 in primary fetal human astroyctes: enhancement of viral gene expression by Nef

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Dementia is a common complication of AIDS which is associated with human immunodeficiency virus type 1 (HIV-1) infection of brain macrophages and microglia. Recent studies have shown that astrocytes are also infected in the brain but HIV-1 replication in these cells is restricted. To determine virus specificity of this restriction we tested the expression of 15 HIV-1 molecular clones in primary human fetal astrocytes by infection and DNA transfection. Infection with cell-free viruses was poorly productive and revealed no clone-specific differences. In contrast, transfected cells produced transiently high levels of HIV-1 p24 core antigen, up to 50 nanograms per ml culture supernatant, and nanogram levels of p24 were detected 3–4 weeks after transfection of some viral clones. The average peak expression of HIV-1 in astrocytes varied as a function of viral clone used by a factor of 15 but the differences and the subsequent virus spread did not correlate with the tropism of the viral clones to T cells or macrophages. Functional *vif*, *vpu*, and *vpr* genes were dispensable for virus replication from transfected DNA, but intact *nef* provided a detectable enhancement of early viral gene expression and promoted maintenance of HIV-1 infection. We conclude that primary astrocytes present no fundamental barriers to moderate expression of different strains of HIV-1 and that the presence of functional Nef is advantageous to virus infection in these cells.

Keywords: HIV-1 tropism; DNA transfection; neuropathogenesis; AIDS

Introduction

The origin of neuropathology associated with HIV-1 infection is complex, with both direct HIV-1 infection of brain cells and indirect effects of products of infected cells contributing to disease (Price *et al*, 1988; Volsky *et al*, 1992; Lipton and Gendelman, 1995). It is generally agreed that macrophages and microglial cells are the major target cells productively infected with HIV-1 in the brain (Sharer, 1992; Stoler *et al*, 1996), but analyses of brain autopsies from AIDS patients by sensitive virus detection assays revealed infection of astrocytes (Saito *et al*, 1994; Tornatore *et al*, 1994a; Ranki *et al*, 1995; Takahashi *et al*, 1996), in some reports in as many as 0.7% of cells in subcortical white matter (Takahashi *et al*, 1996). It is of interest that in most studies infected astrocytes in autopsy tissues contain viral regulatory gene mRNA or protein, but little or no detectable structural gene products (Saito *et al*, 1994; Tornatore *et al*, 1994a), indicating persistent but low-productive infection in this tissue *in vivo*. These findings are consistent with many studies of HIV-1 infection of astrocytes (Cheng-Mayer *et al*, 1987; Tornatore *et al*, 1991; Hatch *et al*, 1994; Nath *et al*, 1995) and glial cell lines in culture (Cheng-Meyer *et al*, 1987; Chiodi *et al*, 1987; Dewhurst *et al*, 1987; Kunsch and Wigdahl, 1989), where restricted, noncytopathic viral expression is the rule. This pattern differs from the typically productive infection in lymphocytes and macrophages and it may be attributed to different biological properties of astroglial cells such as the paucity of surface CD4 receptors (Cheng-Mayer *et al*, 1987) or the presence of cell-

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specific factors governing virus replication (Basagra *et al*, 1992; Tillman *et al*, 1994). Although HIV-1 was shown to enter astroglial cells by a CD4-independent route (Clapham *et al*, 1989; Harouse *et al*, 1989; Chesebro *et al*, 1990; Mizrachi *et al*, 1991), the efficiency of this step in initiation of viral replication has not been fully determined and it may be low. In addition, several reports suggested the existence of intracellular restrictions to HIV-1 expression in astrocytic cells, including down-modulation of proviral DNA transcription (Shahabuddin *et al*, 1992), cellular block in Rev function (Neumann *et al*, 1995), absence of proteolytic processing of envelope precursor glycoproteins (Shahabuddin *et al*, 1996), and failure to accumulate unspliced (but not multiply spliced) viral transcripts, resulting in low levels or absence of viral structural proteins (Tornatore *et al*, 1994b).

HIV-1 infection in astrocytic cells can also be restricted as a function of virus strain as shown in our study using a CD4-positive human glioma H4 cells (Shahabuddin *et al*, 1996). Of nine HIV-1 clones tested only one produced efficient spreading infection in H4/CD4, a defect we subsequently localized to virus strain-specific restriction at the level of gp160 processing (Shahabuddin *et al*, 1996). The virus strain dependence of HIV-1 infection of primary astrocytes is not well understood. Previous studies yielded inconsistent results, with some showing virus strain-specific replication (Cheng-Meyer *et al*, 1987; Nath *et al*, 1995) and others not (Hatch *et al*, 1994). Interpretation of these results is complicated by the highly inefficient infection of astrocytes by cell-free virus preparations used in these studies (Cheng-Meyer *et al*, 1987; Nath *et al*, 1995; Hatch *et al*, 1994). HIV-1 infection of astrocytes can be initiated with greater efficiency by transfection of viral DNA (Tornatore *et al*, 1991, 1994b). In the present work, we used this approach to determine whether HIV-1 expression in astrocytes requires any defined viral characteristics. We tested a set of 15 HIV-1 molecular clones which are well characterized genotypically and phenotypically, some of which contain defects in viral auxillary genes known to modulate HIV-1 expression in primary cells. Representative clones were also tested in a standard cell-free infection. Our results indicate that HIV-1 expression in astrocytes is virus clone-dependent but without clear relation to viral strains of previously defined tropism categories. Moreover, by comparison of clones carrying functional or defective viral auxiliary proteins, we have identified a role for Nef in early viral gene expression in astrocytes.

Results

HIV-1 replication after cell-free virus infection of astrocytes

In the first series of experiments, we attempted to determine the kinetics of HIV-1 infection in astrocytes exposed to cell-free viruses by monitoring the levels of p24 in culture supernatants up to 12 days after infection (Figure 1). In addition to viral clones, we used the well characterized macrophage-tropic HIV-1 strain ADA produced in primary macrophages (Gendelman *et al*, 1988). The cells were infected at 1 picogram of viral p24 per cell (an equivalent of multiplicity of infection of 0.5 as determined previously in appropriate target cells) to maximize virus expression. Figure 1 shows the results of three representative infection experiments using different batches of astrocytes and different virus preparations. Consistent with previous studies (Cheng-Meyer *et al*, 1987; Tornatore *et al*, 1991; Hatch *et al*, 1994), infection of astrocytes with cell-free HIV-1 was highly inefficient and could not be reliably determined by assay of p24 levels. In two experiments shown, the supernatant p24 declined from 0.4–1.2 ng/ml (Figure 1A) or

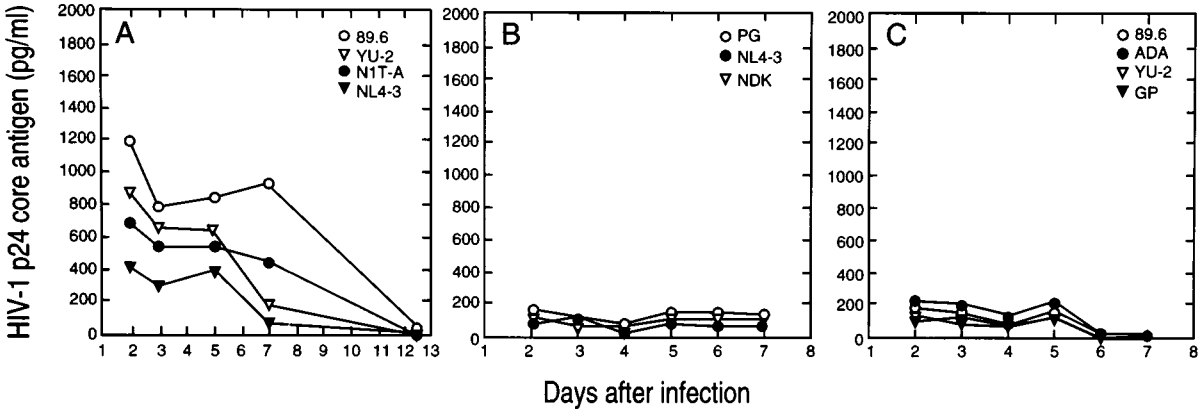

Figure 1 Kinetics of HIV-1 p24 core antigen production by astrocytes infected with cell-free HIV-1. The figure shows three independent experiments using different populations of astrocytes infected with indicated HIV-1 strains. Cells were infected as described in text. p24 was measured in culture supernatants by p24 Elisa assay at the times indicated.

0.1–0.2 ng/ml (Figure 1C) at early times after infection to undetectable levels within 7–12 days after infection. Most of the p24 detected probably represented shedding of the input virus from cells, masking any new viral protein production known to occur at low levels early after infection (Tornatore *et al*, 1991). Background p24 levels only were observed in the third experiment summarized in Figure 1B. With the assay used, we observed no significant differences among the eight viral strains tested by direct infection in their productivity in astrocytes (Figure 1). We conclude that infection of astrocytes with cell-free HIV-1 is too inefficient to determine virus strain-specific requirements for virus expression in these cells by measurement of viral protein levels in culture media. These results do not exclude the possibility that one major block to HIV-1 replication in primary astrocytes is at the stage of virus entry and that none of the strains tested here was able to overcome it.

HIV-1 replication after proviral DNA transfection into astrocytes

We next tested HIV-1 expression in astrocytes transfected with proviral HIV-1 DNA by following the kinetics of p24 antigen in culture supernatants for 3–4 weeks after transfection (Figure 2). In contrast to infection with cell-free viruses (Figure 1), transfections of all viral DNAs tested resulted in efficient p24 production. In all experiments and regardless of the clone used, p24 expression peaked 2–5 days after transfection and p24 levels declined thereafter. However, in the experiments shown in Figure 2A and C, expression of ANL4-3, YU-2, NLHXADA-PG, and NLHXADA-GP continued at relatively high levels for 20 days post-transfection, and YU-2 expression actually increased between day 20 and 25 (Figure 2C). Transient viral expression is the hallmark of HIV-1 infection in astrocytes and the majority of glioma cell lines, regardless of whether infection is initiated by DNA transfection (Dewhurst *et al*, 1987; Srinivasan *et al*, 1988) or exposure to cell free virus (Cheng-Meyer *et al*, 1987; Tornatore *et al*, 1991). We were surprised, however, by the extent of peak p24 production after transfection and by the long term antigen expression in some transfectants (Figure 2). All of the initially tested astrocyte populations produced at least 2 ng p24 per ml at a peak and some, e.g. 89.6 and NL4-3, reached 15 and 50 ng, respectively (Figure 2B). This is significantly more efficient viral protein production than 0.3–1.3 ng per ml reported previously for transfection with NL4-3 DNA (Tornatore *et al*, 1991). It is not obvious why our transfections into astrocytes were more productive than previously reported. The major variables of gestational age of the fetal brain donors, astrocyte preparation, culture conditions, and transfection conditions employed were similar to those previously published (Tornatore *et al*, 1991), and the most productive viral clone tested was NL4-3, the clone used for previous studies (Tornatore *et al*, 1991). One modification of the previous protocol we employed was a DMSO 'shock' at the termination of transfection, which may have facilitated DNA uptake. Similar results of transfection of NL4-3 and Z6 ranging from 20–40 ng p24 per ml were found in experiments in which DMSO was replaced by glycerol for the treatment (not shown), supporting the role of this step in efficient uptake of DNA.

The studies summarized in Figure 2 also indicated that in addition to directing relatively high p24 synthesis in astrocytes after transfection, the HIV-1 clones tested also differed in their peak p24 levels. NL4-3 and 89.6 showed the highest p24 production and NDK, N1T-A, and 282 clones were among the lowest producers. To exclude that differences in DNA uptake, rather than differences in HIV-1 replication, were responsible for the differential p24 expression observed we co-trans-

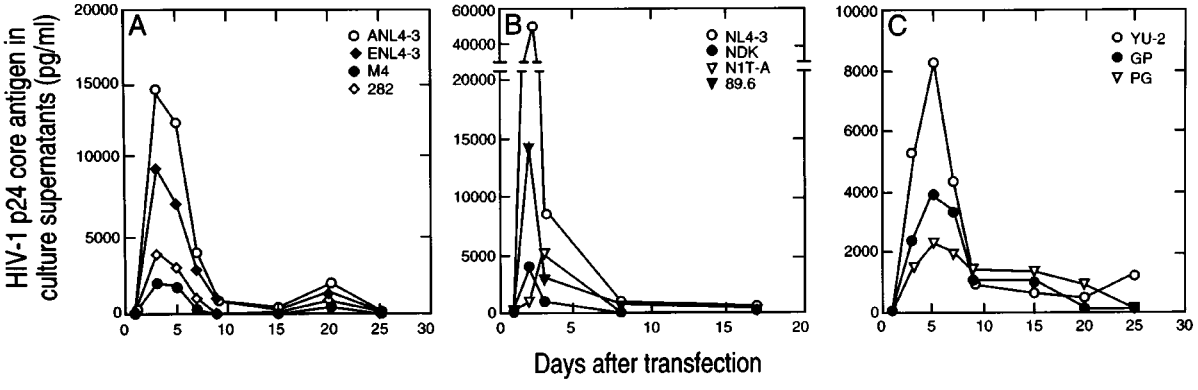

Figure 2 Kinetics of HIV-1 p24 core antigen production by transfected astrocytes. Each panel shows the transfection of a different population of astrocytes with the indicated viral DNAs. Cell supernatants were harvested at the indicated times after transfection and p24 levels were determined by Elisa.

fected DNA encoding HIV-1 and an expression vector of bacterial β-galactosidase, placZ, and determined protein expression by each vector to standardize for transfection efficiency (Table 1). We selected clones producing the highest and the lowest levels of p24 as shown in Figure 2B, NL4-3 and NDK, respectively, and clone Z6 which also was found to replicate well in astrocytes (*vide infra*). When standardized for DNA uptake by the amount of β-galactosidase produced, it is clear that NL4-3 and Z6 replicated in astrocytes about 15 times more efficiently than did NDK, consistent with the findings shown in Figure 2. In contrast, when rhabdomyosarcoma RD cells were used for transfection of NL4-3 and NDK, the clones were equally productive, producing about 100 ng p24 per ml culture medium 3 days after transfection. These results indicated that host cell-virus specific interactions account for the differences in viral protein production observed in HIV-1 clones expressed in astrocytes, since the virus clones themselves are equally replication competent in other cell types.

To investigate further whether the observed virus clone-specific expression in astrocyte can be generalized and whether viral auxiliary genes contribute to these differences, we performed a survey including 16 transfection experiments using 16 different populations of astrocytes and up to nine different proviral DNAs per experiment and evaluated p24 levels in culture supernatants 3–5 days after transfection, at the peak of the antigen production as determined in studies summarized in Figure 2. We used 13 different HIV-1 clones in these experiments, the clones were of North American or African origin and they represented the full range of known HIV-1 tropisms. Some of the clones carried one or more inactive viral auxiliary genes, which are important in viral replication in primary lymphocytes and macrophages (Gabuzda *et al*, 1994; Connor *et al*, 1995; reviewed in Trono, 1995), but their effects on HIV-1 expression in primary astrocytes are unknown. The results of the survey are shown in Table 2. There was significant variability in the expression of individual viral clones in repeated transfections, probably reflecting the differences among astrocyte batches and individual transfections. However, the large number of experiments permitted us to stratify HIV-1 clones according to their average peak expression in astrocytes. Generally, all the viral clones tested replicated in astrocytes to some extent but they differed by up to 15-fold in their average peak p24 production. The differences in virus replication were virus-clone specific but did not assort clearly with cellular tropism of the clones or presence or absence of functional auxiliary genes *vpu*, *vpr*, and *vif* (Table 2). For example, the T cell line tropic NL4-3 and NIT-A were the highest and the lowest producers, respectively, while macrophage-tropic clones NLHXADA-GP and YU-2 replicated in the middle of the range determined for T cell line tropic clones. The isogenic NLHXADA-GP and -PG, which differ only in their M- or T-tropism-conferring V3 regions (Westervelt *et al*, 1991) replicated similarly at the peak of infection (Table 2) and during 3 weeks in culture (Figure 2C), indicating that neither M- nor T-tropism confers an advantage for HIV-1 expression and spread in astrocytes. Similarly, the *vpu* and *vpr*-negative NIT-A replicated poorly in astrocytes but Z6, which is also defective in both of these genes, was highly productive (Table 2). Although NIT-A and Z6 have different genetic backgrounds, these data suggest that *vpu* and *vpr* are dispensible for HIV-1 replication *per se* in astrocytes. Somewhat different results were obtained after repeated transfections of a set of isogeneic *vif*-defective clones ANL4-3, ENL4-3, and M4NL4-3 (Simm *et al*, 1995; Sova *et al*, 1997). The wildtype ANL4-3 and its vif-negative mutant ENL4-3, which expresses only N-terminal 38 amino acids of Vif (Sakai *et al*, 1991; Sova *et al*, 1997), produced comparable amounts of p24 (Table 2), indicating that Vif is also dispensible in this system. This result is consistent with previous reports which showed that Vif does not affect HIV-1 expression from proviral DNA but is required for HIV-1 spread in lymphocytes and macrophages (von Schwedler *et al*, 1993; Gabuzda *et al*, 1994; Simm *et al*, 1995; Chowdhury *et al*, 1996). However, M4NL4-3, another isogenic construct in which the essential cysteines in Vif were replaced by leucines (Sova *et al*, 1997), was very poorly productive. Our results indicate that Vif function in astrocytes may rely upon the essential cysteines for a distinct aspect of virus expression involving p24 production, however this preliminary observation requires further study.

Table 1 Expression of HIV-1 transfected DNA in primary astrocytes standardized for transfection efficiency.

HIV-1 clone	*HIV-1 expression (ng p24/ 10^6 cells)*	*pLacZ expression (pg β-gal/μg protein)*	*ng intracellular p24/pg β-gal/μg protein*
NL4-3	270	19	14.2
Z6	480	28	16.9
NDK	66	75	0.9

Astrocytes were transfected with a 10:1 mixture of HIV-DNA and pLacZ DNA and evaluated for HIV-1 and β-galactosidase expression 3 days after transfection as described in Materials and methods.

The effects of Nef upon HIV-1 replication in astrocytes

Previous studies have indicated that HIV-1 infected astrocytes express higher levels of Nef than of viral structural proteins (Tornatore *et al*, 1994a,b), suggesting that Nef plays a distinct role in HIV-1

Table 2 Expression of HIV-1 molecular clones in primary human astrocytes.

Virus clone	*Tropism*	*Inactive genes*	*Peak supernatant p24 core antigen levels (ng p24/ml)*																*Average (ng p24/ml)*	
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*		
N1T-A	T	*vpr, vpu*				0.9	1.5	0.5											1.0	*n*=3
HXB-2	T	*vpr, vpu nef*	1.8																1.8	*n*=1
M4NL4-3	T	*vif*			2.1	3.4		0.7		4.8		1.2					1.0	0.1	1.9	*n*=7
NDK	T	none		1.4		4.0	1.5			3.3			2.2	1.8	0.7	2.5			2.4	*n*=9
KS282	T	*vpr, vpu, vif*			4.0			1.8	6.2	2.8		0.6				3.2	0		2.7	*n*=7
PG	T	*vpr*			2.3	3.0	4.9	0.7	6.2										2.7	*n*=4
GP	M	*vpr*			3.9														3.9	*n*=1
YU-2	M	none			8.3	6.5	10.1	2.2		5.7	1.1				1.9	3.0			4.4	*n*=9
ENL4-3	T	*vif*		1.6	9.6	4.8	7.1			6.4		2.1	7.0		1.9		5.1	7.1	5.3	*n*=10
89.6	M/T	none	12.1			14.1	5.4			2.8	6.4	0.5		0.3	10.3	1.8			6.0	*n*=9
ANL4-3	T	none		3.6	14.8	22.4	1.9			5.4		0.6	8.5		2.9		3.8	1.9	6.6	*n*=10
Z6	T	*vpr vpu*		2.1			15.8	40.0	6.4			6.5		2.4		4.1		1.7	9.9	*n*=8
NL4-3	T	none	15.0		50.8		24.1	0.7		22.0	6.3	3.7	15.1	6.4	13.7		3.1		14.6	*n*=11

Fetal primary astrocytes were transfected with viral DNAs and tested for p24 antigen levels in culture supernatants 3–5 days after transfection as described in Materials and methods. Results of 16 independent transfection experiments with indicated viral DNAs are shown. The viral clones are described in the following references: NIT-A, Sakai *et al*, 1988; HXB-2 Shaw *et al*, 1984; M4NL4.3, Sova *et al*, 1997; NDK, Spire *et al*, 1989; KS282, Sakai *et al*, 1991; PG and GP, Westervelt *et al*, 1991; YU-2, Li *et al*, 1991; ENL4-3 and ANL4-3, Simm *et al*, 1995; 89.6, Collman *et al*, 1992; Z6, Srinivasan *et al*, 1987, and NL4-3, Adachi *et al*, 1986.

replication in astrocytes. To evaluate the role of Nef directly, we compared the expression of isogenic Nef-positive and Nef-negative HIV-1 clones NL-P1Nef$^+$ and NL-P1Nef$^-$ in astrocytes using cell-free virus infection and transfection. The clones are based on NL4-3 proviral DNA constructed to carry the marker gene human placental alkaline phosphatase (PLAP) which is expressed as a multiply spliced messenger RNA, similar to the early viral genes (Collins *et al*, 1998). PLAP can be detected using indirect immunofluorescent staining as a measure of early gene expression (Collins *et al*, 1998). Transfection of NL-P1Nef$^+$ and NL-P1Nef$^-$ HIV-1 into astrocytes yielded similar kinetics of p24 production (Figure 3A), indicating that similar to other HIV-1 auxiliary genes (Table 2), Nef has no effect on HIV-1 expression from transfected DNA. A different result was obtained when requirement for Nef was tested during infection with cell-free virus (Figure 3B). p24 expression after infection of astrocytes, representing both the input virus inoculum and any newly synthesized p24, was similar for Nef$^+$ and Nef$^-$ viruses early after infection, but then declined faster in Nef$^-$ compared to Nef$^+$ virus infection (Figure 3B). To determine whether the observed difference in p24 levels represented new protein synthesis, infected cells were examined for expression of the marker PLAP, which unlike p24, is not carried over with the input virus (Figure 4 and Table 3). We found that Nef$^+$ virus expressed considerably more PLAP and over longer time period than did Nef$^-$ virus (Figure 4 and Table 3). Consistent with previous reports (He *et al*, 1997a), infection of primary astrocytes with HIV-1 as

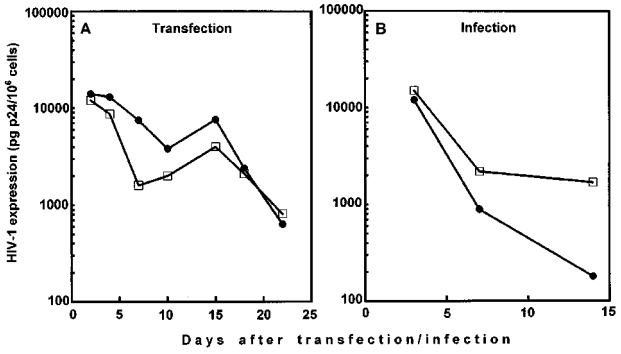

Figure 3 Expression of Nef$^+$ and Nef$^-$ HIV-1 in astrocytes after proviral DNA transfection or cell-free virus infection. Astrocytes were either (**A**) transfected with NL-P1Nef$^+$ or Nef$^-$ viral DNA or (**B**) infected with cell-free Nef$^+$ or Nef$^-$ viruses, and virus production was determined by measurement of intracellular p24 as described in Materials and methods.

followed by PLAP expression was inefficient, but the PLAP marker was still detectable at low levels 15 days after infection with Nef$^+$ virus (Table 3). These results indicate that Nef increases expression of early HIV-1 genes in astrocytes (represented by PLAP in NL-P1 recombinants) and contributes to maintenance of HIV-1 infection in these cells.

Discussion

We have compared HIV-1 expression in primary human astrocytes after infection and transfection

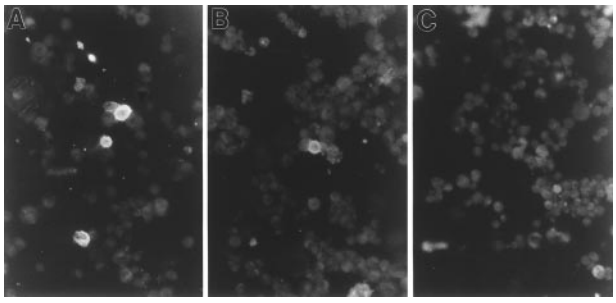

Figure 4 *In situ* detection of HIV-1 expression in infected astrocytes by PLAP staining. Astrocytes were infected with HIV-1 PLAP reporter viruses NL-P1Nef$^+$ and Nef$^-$, the cells were fixed in acetone 7 days after infection and stained for PLAP by indirect immunofluorescence as described. (**A**) and (**B**): PLAP IF of Nef$^+$ virus-infected cells in two different fluorescence microscope fields; (**C**): a field of Nef$^-$ virus-infected cells.

Table 3 Difference in infection of astrocytes by Nef$^+$ and Nef$^-$ HIV-1 as determined by expression of a marker gene human placental alkaline phosphatase (PLAP).

Time after infection (days)	*Percent of PLAP-positive cells*	
	NLP1 Nef$^+$	*NLP1 Nef$^-$*
3	0.33	<0.1
7	0.5	<0.1
15	0.2	0

Infected cells were stained for PLAP by indirect immunofluorescence as described in Materials and methods and evaluated under a fluorescence microscope (see also Figure 4). At least one thousand cells per smear were counted to determine the frequency of positive cells listed in the Table.

with a large panel of well-defined molecular clones of the virus. Consistent with previous reports (Cheng-Mayer *et al*, 1987; Tornatore *et al*, 1991; Hatch *et al*, 1994), infection with cell-free HIV1-was inefficient but DNA transfection permitted virus replication, in some cases at surprisingly high levels (Table 2). However, none of HIV-1 clones tested here, including high p24 expressors (Figure 2, Table 2), was able to fully overcome the astrocytic block to HIV-1 infection and establish a highly productive, spreading infection similar to that in other primary host cells of HIV-1. Since all the clones were significantly more productive after DNA transfection than after cell-free virus infection (Figure 1 *versus* Figure 2), our data indicate that the major restriction of HIV-1 infection in primary astrocytes is at early stages of the virus replicative cycle, such as entry, uncoating, or reverse transcription. Low efficiency of these steps is also indicated by quantitative studies using pseudotyped reporter HIV-1 expressing green fluorescence protein (He *et al*, 1997a) and HIV-1 recombinants expressing human placental alkaline phosphatase expressed as an early viral gene product (Table 3). Limited entry of HIV-1 into astrocytes may in part be attributed to the absence of the primary HIV-1 receptor, CD4, on these cells (Cheng-Mayer *et al*, 1987). Although astrocytes likely display some viral receptors which permit the low-level infection observed in these cells, only cells expressing both CD4 and one or more of the recently identified fusion co-receptors are susceptible to efficient productive infection by HIV-1 (Littman, 1998). We have demonstrated this requirement in glioblastoma cells H4, which are CD4-negative and poorly infectible by HIV-1 but can be converted to highly susceptible hosts for the virus by stable expression of a CD4 encoding vector (Volsky *et al*, 1992). The HIV-1 receptor and co-receptors which are functional on primary astrocytes are not known and may prove to be the most important variables in understanding restrictions in viral replication in these cells.

Previous reports suggested that HIV-1 infection of astrocytes can also be restricted after reverse transcription, at the level of synthesis of viral structural but not regulatory proteins (Tornatore *et al*, 1991, 1994b). The observed transient expression of p24 in transfected astrocytes (Figures 2 and 3A) is consistent both with the proposed downregulation of the late stages of virus replication (Tornatore *et al*, 1994b) and with limited spread of progeny virus due to block at entry. It is noteable that with our transfection approach all the HIV-1 clones tested expressed p24 at relatively high levels, up to 50 ng per ml of culture supernatant, and in some cases significant p24 production lasted for at least 3 weeks in culture (Figure 2 and 3A). This indicates that once inefficient entry of HIV-1 is bypassed, human astrocytes can support persistent production of viral structural proteins and virus replication. Our data also indicate that astrocytes can support replication of a wide range of HIV-1 strains, of both North American and African origin, of tropism to either T cells or macrophages, and of *vif*, *vpu*, or *vpr* positive and negative phenotypes. Glial cells (astrocytes and oligodendrocytes) make up 50% of the total cell number in the brain. Although our data (Figure 4 and Table 3) and results of others (He *et al*, 1997a; Takahashi *et al*, 1996) indicate that only a small proportion of astrocytes (0.5–4%) can be infected by HIV-1 *in vitro* and *in vivo*, the large total number of astrocytes in the brain, their apparent broad susceptibility to HIV-1 infection, and their ability to support virus replication all suggest that astrocytes can constitute a significant reservoir of HIV-1 in the brain.

The studies described here also revealed for the first time a potential role of Nef in HIV-1 infection in astrocytes. Previous reports have shown that HIV-1

infected astrocytes examined in autopsy preferentially express Nef mRNA and protein rather than structural viral products (Saito *et al*, 1994; Tornatore *et al*, 1994a). Preferential expression of multiply spliced versus unspliced transcripts was also reported in astrocytes infected in culture (Tornatore *et al*, 1994b). The contribution of Nef to these effects has not been directly examined. Using isogenic Nef-positive and negative HIV-1 marker clones we found that Nef increases the production of a marker protein PLAP expressed from a doubly-spliced mRNA as part of HIV-1 genome (Figure 4 and Table 3), indicating that Nef is required for efficient expression of early viral genes. Although there was little effect of Nef upon structural gene expression when replication was initiated by transfection (Figure 3A), Nef also appeared to promote the maintenance of HIV-1 in astrocytes after infection with cell-free virus (Figure 3B). Together, the present findings, the elevated expression of Nef in infected astrocytes (Tornatore *et al*, 1994b), and the recent demonstration that Nef transactivates the protooncogene c-kit in infected astrocytes (He *et al*, 1997b), all indicate that Nef plays a unique role during HIV-1 replication in astrocytes and perhaps in neuropathogenesis. Further studies are ongoing to determine the phase of HIV-1 replication in astrocytes in which Nef is active.

In summary, these studies suggest that primary astrocytes present no fundamental barriers to moderate expression of different strains of HIV-1 *in vitro* and that Nef facilitates early viral gene expression and may promote maintenance of viral infection in astrocytes. The presence of viral DNA and regulatory proteins in astrocytes of some HIV-1 infected children and adults indicates that astrocytes can be infected *in vivo* (Saito *et al*, 1994; Tornatore *et al*, 1994a; Takahashi *et al*, 1996). The source of the virus infecting astrocytes in the brain is unknown, but these and other studies (He *et al*, 1997a) suggest that it may originate either from macrophages or T lymphocytes. Moreover, as we recently reported, HIV-1 may adapt to growth in astrocytes *in vivo* (Canki *et al*, 1997) and other reports suggest that HIV-1 interaction with astrocytes may affect astrocyte functions (Benos *et al*, 1994; He *et al*, 1997b). The systems we describe here permit direct investigation of the range of expression of HIV-1 strains in primary astrocytes and the impact of this replication on cellular function and neuropathogenesis.

Materials and methods

Cells

Fetal astrocytes were isolated as previously described (Canki *et al*, 1997) from brains of 12–22 week fetuses obtained from elective abortions in full compliance with NIH guidelines. Brain tissue composed of telencephalon with both cortical and ventricular surfaces was dissected and mechanically dissociated and then passed through sieves of 230 μm and 140 μm. Cells were permitted to adhere for several days and adherent cells were verified as astrocytes by staining with fluorescein conjugated anti-glial fibrillary acidic protein (GFAP) (Dako, Palo Alto, CA, USA). Astrocytes were cultured in Dulbecco's modified Eagles' medium supplemented with 10% fetal bovine serum at 2×10^6 cells per 100 mm dish. Early passage astrocytes, which were verified as 99% GFAP positive, were infected with cell-free HIV-1 or by transfection of cloned viral DNA as described.

Viruses and molecular clones

The viral clones used in this work, their predominant tropism, and the functional status of viral auxiliary genes are listed in Table 1. The clones include T cell line-tropic NIT-A, HXB2, NDK, NLHXADA-PG, Z6, NL4-3; macrophage-tropic YU-2 and NLHXADA-GP, and dual tropic 89.6. The references describing these clones are also listed in Table 1. For determination of the role of Nef in HIV-1 infection of astrocytes we used two derivatives of NL4-3 which carry the marker gene human placental alkaline phosphatase (PLAP) in the presence or absence of Nef (Collins *et al*, 1998). The pSV-LacZ plasmid used for cotransfection with viral DNA in some experiments was obtained from Promega (Madison, WI). All plasmids were amplified in *E. coli* and their DNA was isolated and purified according to standard protocols as previously described (Ausubel *et al*, 1989; Chowdhury *et al*, 1996).

Transfection and infection

Transfection was performed using 0.5–2 μg plasmid DNA per 10^6 cells and calcium phosphate precipitation by standard methods (Ausubel *et al*, 1989) with one modification: 4 hours following addition of DNA to astrocytes, cells were exposed to 10% DMSO in Dulbecco's modified Eagle medium for 5 min and washed prior to culture. For cell-free HIV-1 infection experiments, selected viral clones were transfected by the above procedure into RD or 293T cells; 3–4 days after transfection, culture supernatants were collected, virus was sedimented by centrifugation, and concentrated viral stocks were standardized for their HIV-1 p24 core antigen content and used for infection of astrocytes by standard procedures (Canki *et al*, 1997).

Evaluation of HIV-1 expression

For detection of HIV-1 protein, cell supernatants were harvested as indicated for assay of extracellular p24 core antigen using the HIV Ag kit according to the manufacturer's instructions (Coulter, Hialeah, FL, USA). In addition, HIV-1

antigen expression by single cells was monitored by indirect immunofluorescent staining using AIDS sera staining acetone fixed cells. For evaluation of viruses carrying the human PLAP gene, we also performed indirect immunofluorescent staining using a mouse monoclonal anti-PLAP (Serotec, Raleigh, NC, USA).

Evaluation of β-galactosidase expression
Cell lysates were prepared in 1% nonidet P-40 in phosphate buffered saline and assayed for *β*-galactosidase-mediated cleavage of chlorophenol red *β*-galactopyranoside spectrophotometrically as previously described (Nussbaum *et al*, 1994). *β*-galactosidase activity is expressed relative to total protein content of cell lysates as determined in a Biorad micro protein assay (Biorad, Hercules CA, USA).

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