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Short Communication

Transient infection of astrocytes with HIV-1 primary isolates derived from patients with and without AIDS dementia complex

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We have studied the replication capacity of primary HIV-1 isolates obtained from four AIDS patients in astrocytes. Two patients (P1 and P2) had neurological manifestations without AIDS Dementia Complex (ADC). The other two patients (P3 and P4) had ADC. Two astrocytoma cell lines and normal fetal astrocytes were incoculated with each of these four viral isolates. Viral DNA and mRNA synthesis and also protein accumulation were followed at various times after infection. We found that tumoral as well as fetal astrocytes were susceptible to HIV-1 infection. Three of four viral isolates (P2, P3, P4) were able to infect astrocytes. Both ADC viral isolates (P3, P4) infected astrocytes with identical transcriptional patterns: rev, nef and unspliced mRNAs were expressed for 2 days after infection. The non-ADC patient (P2) with the isolate leading to viral replication in astrocytes had an HIV-1 associated multifocal demyelinating neuropathy. In this case, only nef and unspliced mRNAs were detected a few days after virus inoculation. In all cases, infection of astrocytes was transient and the level of unspliced mRNAs in infected astrocytes was lower than in chronically HIV-1 infected T cells. More extensive work would allow a better understanding of the role of astrocytes in ADC.

Keywords: Polymerase chain-reaction; viral messenger-RNA quantitation; nef-protein; central-nervous-system

AIDS dementia complex (ADC) is a frequent neurological complication associated with HIV-1 infection. Neuropathological studies have demonstrated that macrophages and microglial cells are the main targets of HIV-1 in the brain (Koenig *et al*, 1986, Wiley *et al*, 1986). Both cell types are susceptible to highly productive HIV-1 infections *in vivo* (Koenig *et al*, 1986, Wiley *et al*, 1986, Nuovo *et al*, 1994, Bagasra *et al*, 1996). There is increasing evidence to suggest that HIV-1 can infect cells of neuroectodermal origin, including astrocytes (Nuovo *et al*, 1994, Tornatore *et al*, 1994b, Saito *et al*, 1994, Ranki *et al*, 1995, Bagasra *et al*, 1996). It is thought that HIV-1 infection of astrocytes could contribute directly to the nervous system damage. In the present study, we have investigated the ability of primary HIV-1 isolates obtained from patients with and without ADC to replicate in astrocytes.

Normal human fetal astrocytes (Novagen, San Diego, CA) and two human astrocytoma cell lines, MOG-G-UVM and CCF-STTG1 (European Collection of Animal Cell Cultures) were inoculated with viral isolates derived from non-ADC and ADC patients. All these cell types were glial fibrillary acidic protein (GFAP)-positive and CD4-negative (data not shown). Among the four viral isolates studied, two derived from patients without ADC (P1: cerebral toxoplasmosis and P2: HIV-1-associated multifocal demyelinating neuropathy) and two derived from patients with ADC (P3 and P4). Viral isolates were derived from the inoculation of patients plasma into 3-day-old PHA-stimulated donor PBMC. Expanded viral stocks were obtained as previously described (WHO network for HIV isolation and characterization, 1994) and the peak of reverse transcriptase (RT) activity was determined for each isolate. The genetic and biological

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analysis of these isolates was described elsewhere (Brengel-Pesce *et al*, 1996). One of the ADC viral isolates (P4) had a subtype D genotype in contrast with the three other isolates which had a subtype B genotype. P1 and P4 viral isolates had an SI phenotype whereas P2 and P3 isolates had an NSI phenotype in MT-2 cells (Brengel-Pesce *et al*, 1996). Inocula were standardized by RT activity: 10 000 c.p.m. of viral isolates at the peak of RT activity were added per 10^6 cells. Each culture flask was incubated with the virus diluted in culture medium (DMEM/10%FBS) at 37°C for 16 h. As a control, astrocyte inoculations with various quantities of free virus (up to 30 000 c.p.m./10^6 cells) were performed, and led to the same kinetics of infection. All four viral isolates were tested in human astrocytoma cell lines but only the ADC subtype D viral isolate was cultured into normal human fetal astrocytes. In parallel, PHA-stimulated PBMCs were incoculated with the various isolates as a control for biological activity. Culture supernatants were taken at various time points following infection and assayed for p24 antigen capture ELISA (p24 antigen vironostika, Organon-Teknika, Boxtel, Holland) and RT activity. Productive infection was observed in stimulated PBMCs at a time when astrocyte culture supernatants were p24 antigen negative and displayed no RT activity: this suggests either a non productive infection or production below the detection threshold of our methods.

To investigate the characteristics of astrocyte infection, the viral replication cycle was studied at the level of DNA, mRNAs and proteins. After 16 h of cell-virus contact (time D0), and one, two and several days post-infection, cells were scraped, washed three times in PBS and stored at −80°C. Viral DNA was analysed by PCR using 1 μg of extracted DNA and p3/p4 primer pair (*pol* gene) (Table 1). PCR conditions were as previously described and the sensitivity of PCR-detection of viral DNA with p3/p4 *pol* primers is one copy per 1 μg of DNA (i.e. 150 000 cells) after hybridization with the radiolabeled probe (Laure *et al*. 1988). DNA PCR efficiency was tested with β-globin gene primers (Innis *et al*, 1990) (Table 1). No significant difference in HIV-1 infection was seen between astrocytoma cell lines and normal fetal astrocytes (Table 2). Infection with the P4 viral isolate led to similar viral DNA detection in the three cell types. The duration of infection varied between different viral isolates (Table 2). No specific viral DNA could be detected in MOG-G-UVM astrocytes infected with the non-ADC P1 viral isolate. These negative results were confirmed by a positive β-globin PCR. However, viral DNA was detected in CCF-STTG1 astrocytes infected with this P1 strain at time D0 (after 16 h of virus/cell contact). Astrocytes of the CCF-STTG1 cell line appeared to be more susceptible to HIV-1 infection than those of the MOG-G-UVM cell line. P2 (non-ADC) and P3 (ADC) patients presented the same kinetics of infection in both cell lines: a positive PCR was only found at D0 in the MOG-G-UVM cell line and at D1 in the CCF-STTG1 cell line. The ADC P4 viral isolate with a subtype D genotype displayed the most long-lived infection. Viral DNA was present at D3 in both cell lines and still at D6 in fetal astrocytes.

Table 1 Oligonucleotide primers used in this study

Primers	*Sequence 5′-> 3′*	*nct position*[a] *(on HXB2 DNA)*	*Reference*
Pol région			
p3 (1)	TGGGAAGTTCAATTAGGAATACCAC	2810–2834	Laure *et al.* (1988)
p4 (2)	CCTACATACAAATCATCCATGTATT	3116–3093	
Multi-spliced mRNAs			
SK29 (1)	ACTAGGGAACCCACTGCT	501–518	Ou *et al.* (1988)
Exon2 (1)	TGGAAAGGACCAGCAAAG	4931–4948	
Exon3 (1)	GTGTGAATATCAAGCAGG	5435–5452	Schwartz *et al.* (1990)
2606S (1)	GGTGTCGACATAGCAGAATAGGC	5782–5804	
Co12 (1)	CTTAGGCATCTCCTATGGCA	5955–5974	
SV240 (1)	GAAGAAGCGGAGACAGCGACGAAGAGCT	5976–6003	[b]
SV243 (2)	AGGCTCCGCAGATCGTCCCAGATAAGTG	8511–8484	
SV241 (2)	GTCTCTAAGCGGTGGTAGCTGAAGAG	8542–8516	
β-globin			
KM29 (1)	GGTTGGCCAATCTACTCCCAGG		Innis *et al.* (1990)
RS42 (2)	GCTCACTCAGTGTGGCAAAG		
Aldolase mRNA			
1179 (1)	CCCCTTCCGAGGCTAAATCG		[c]
1178 (1)	CTGGTAGTAGCAAGTTCCTGGCAC		

(1) sense; (2) antisense.
[a]Nucleotide positions of primers were determined from HIV-1HXB2 strain DNA.
[b]Primers kindly provided by S Sauvaigo, CisBio, Gif-sur-Yvette, France.
[c]Primers kindly provided by H Perron, UMR 103 Biomérieux SA, Lyon, France.

Table 2 Kinetics of viral DNA detection in infected astrocytes with different HIV-1 isolates

Cells	*Viral isolates*	*Viral DNA detection*						
		D0	*D1*	*D2*	*D3*	*D6*	*D13*	*D28*[a]
MOG-G-UVM[e]	Control (−)[b]	−	−	−	−	nt[c]	−	−
	non-ADC P1	−	−	−	−	nt	−	−
	non-ADC P2	+	−	−	−	nt	−	−
	ADC P3	+	−	−	−	nt	−	−
	ADC P4	+	+	+	+w[d]	nt	−	−
CCF-STTG1[e]	Control (−)	−	−	−	nt	−	−	nt
	non-ADC P1	+	−	−	nt	−	−	nt
	non-ADC P2	+	+	−	nt	−	−	nt
	ADC P3	+	+	−	nt	−	−	nt
	ADC P4	+	+	+	nt	−	−	nt
NHA[f]	ADC P4	+	nt	+	nt	+w	−	−

[a]D0–D28: days after infection; D0: time after 16 h of virus/cell contact.
[b]Control (−) corresponded to mock-infected cells treated in the same conditions as infected cells.
[c]nt: not tested.
[d]+w indicates a weak positive result in PCR after hybridization with radioactive specific probe.
[e]Astrocytoma cell line.
[f]Normal human astrocytes.

Inoculation of MOG-G-UVM astrocytes with the HIV-1_{BaL} strain under the same conditions also led to a transient infection (data not shown). Therefore, although astrocytes were infected by free HIV-1 viruses, and viral RNA was reverse transcribed in these cells, viral DNA was detected only within the first days after virus inoculation suggesting that infection of astrocytes by HIV-1 isolates was transient. To determine the transcriptional activity of the viral DNA present in astrocytes, we looked for multi-spliced and unspliced mRNAs using CCF-STTG1 culture assays (Table 3). PolyA-positive RNAs were extracted from 10^6 astrocytes using oligo-dT dynabeads (Dynal, Oslo, Norway) according to the manufacturers' instructions and eluted in 50 μl of DEPC-treated water and 50 U of RNasin (Promega, France). First, multi-spliced mRNAs were detected by RT–PCR in one step on 10 μl of DNase-treated RNA extract in a total volume of 100 μl. The reaction mixture for RT–PCR contained 50 mM KCL, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 250 μM each dNTP, 40 pmol of SK29 and SV241 primer (Table 1), 10 U of AMV-RT (Boehringer Mannheim, France), and 2.5 U of Taq polymerase (Boehringer Mannheim). The synthesis of cDNA was carried out at 42°C for 2 h, followed by a denaturation at 94°C for 5 min and PCR amplification for 40 cycles (1 min-94°C, 1 min-55°C, 2 min-72°C). All small multi-spliced mRNAs were then amplified: 1 μl of PCR product was re-amplified with mRNA-specific primers (sense primer and SV243) using a nested-PCR for 30 cycles (Table 1). RT–PCR efficiency was tested with primers of aldolase mRNAs (Table 1).

Multi-spliced mRNAs were present only for a few hours after infection (at D0 only) (data not shown). Specific primers were then used to discriminate between the multi-spliced mRNAs expressed at this time point in infected astrocytes (Table 3). There was no mRNA detected in astrocytes infected with the P1 viral isolate. This isolate is unable to infect astrocytes despite a DNA signal at D0 in the CCF-STTG1 cell line. The positive DNA result just after the virus/cell contact may be due to viral adsorption on cells. Moreover, all time points were DNA negative in the MOG-G-UVM cell line. It is interesting to note that HIV-1 viral isolates derived from the two ADC patients (P3 and P4) have the same transcription pattern. Infection with these isolates showed rev/nef and nef transcripts. Conversely, the inoculation of the non-ADC P2 isolate on astrocytes only induced the synthesis of nef transcripts with the small exon 2. Exon 3 and tat mRNAs were not detected in astrocytes infected with either viral isolate.

HIV-1 unspliced mRNA expression was then measured by a quantitative RT–PCR technique adapted by the 'Agence Nationale de la Recherche sur le SIDA' (ANRS) from the Amplicor HIV-1 Monitor test (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA). 10 μl of mRNA extract were treated with 40 U of DNase (Boehringer Mannheim) and 1.5 μl of 10 × buffer (TrisHCl 100 mM pH7.4, $MgCl_2$ 50 mM, KCl 500 mM, $CaCl_2$ 5 mM) for 1 h at 37°C then 5 min at 80°C. The RT–PCR was carried out on 50 000 cells amplifying a fragment in the *gag* gene. Conditions of amplification and PCR products' revelation were fixed by the manufacturers of the Amplicor HIV-1 Monitor test (Roche Diagnostic Systems). A direct PCR, without the RT step, under the same conditions allowed us to verify the DNase efficiency.

Isolates from both ADC patients (P3 and P4) led to the transient expression of a similar quantity of

Table 3 Characterization of multi-spliced mRNAs expressed at time D0 in astrocytes of the CCF-STTG1 cell line infected with different HIV-1 isolates

	mRNAs and nested-PCR primers				
Viral isolates	*tat, rev, nef, (SV240/SV243)*	*tat (2606S/SV243)*	*rev/nef (Co12/SV243)*	*exon2 (Ex2/SV243)*	*exon3 (Ex3/SV243)*
Control (−)[a]	−	−	−	−	−
non-ADC P1	−	−	−	−	−
non-ADC P2	+	−	−	+	−
ADC P3	+	−	+	−	−
ADC P4	+	−	+	−	−

[a]Control (−) corresponded to mock-infected cells treated in the same conditions as infected cells.

Table 4 Unspliced mRNA quantitation in astrocytes of the CCG-STTG1 cell line infected with different HIV-1 isolates

	Quantitation of unspliced mRNAs (copy number/ 10^6 cells)		
Viral isolates	*D0*	*D1*	*D2*[a]
Control (−)[b]	−[c]	−	−
non-ADC P1	nt[d]	nt	nt
non-ADC P2	72431	nt	468
ADC P3	3035	nt	−
ADC P4	4142	37	−

[a]D0–D2: days after infection.
[b]Control (−) was mock-infected cells treated in the same conditions as infected cells.
[c]Cut-off was fixed at OD=0,100 (negative result corresponded to a copy number <25 per 10^6 cells).
[d]nt: not tested.

unspliced mRNAs (Table 4). At time D0, about 3000 and 4000 mRNA copies per 10^6 cells were measured respectively, whereas at D1, mRNA expression fell dramatically. The non-ADC P2 culture strain showed a greater and consequently a more durable expression of unspliced mRNAs in infected astrocytes. Approximately 72 000 mRNA copies were found at D0 and mRNAs persisted at D2. In parallel, mRNAs of cells from the 8E5 T cell line chronically infected with HIV-1 (Gendelman *et al*, 1987) were extracted and unspliced mRNAs were quantitated under the same conditions. We found about 520 000 unspliced mRNA copies per 10^6 cells. The quantities of expressed mRNAs in astrocytes were seven to 120 times lower than in these T cells. These results indicate that astrocytes can be infected with non-ADC and ADC HIV-1 isolates with a transient expression of early and late mRNAs.

HIV-1 restricted infection of astrocytes was previously associated with an accumulation of Nef proteins in these cells (Tornatore *et al*, 1994b, Saito *et al*, 1994, Ranki *et al*, 1995). We looked for the presence in CCF-STTG1 astrocytes of Nef and p24 proteins, using indirect immunofluorescence and Western-blotting. Mock-infected astrocytes were used as negative controls and T-cells of the 8E5 cell line as positive controls. Monoclonal antibodies used were directed against Nef protein (Chemicon International, Temecula, CA) and p24 protein (Biodesign International, Kennebunk, ME). For Western-blotting, 3.10^6 HIV-1 infected or uninfected cell pellets were lysed with 200 μl of sucrose 10%, EDTA 2 mM, SDS 2%, PMSF 2 mM, Tris HCl 62.5 mM (pH 6.8). Samples were incubated for 5 min at 100°C and 5% mercaptoethanol, 0.01% pyronine G was added. Proteins (10^6 cells) were separated on a 12.5% polyacrylamide gel (SDS–PAGE) and transferred to nitrocellulose (Amersham International plc, Buckinghamshire, England) by electro-blotting according to the manufacturer's instructions (Sartoblot II-S; Sartorius, Palaiseau, France). Immunoblotting was performed in PBS/5% nonfat dried milk. Nitrocellulose was incubated overnight with Nef and p24 monoclonal antibodies and a human HIV-1 polyclonal serum. After washing with PBS/0.05% Tween 20, the second antibody (antimouse or antihuman) was added. Detection was by chemiluminescence (ECL Western-blotting detection reagents, Amersham).

Mock-infected astrocytes showed no positive signal with the indirect immunofluorescence assay, using either Nef or p24 monoclonal antibody. Astrocytes inoculated with non-ADC P1 and P2 isolates remained negative to both antibodies. Astrocytes infected with the single ADC P4 isolate exhibited a weak positive signal with Nef antibody, in particular in the nucleus. Western-blotting assay did not confirm these results, since viral proteins were detected neither with Nef and p24 monoclonal antibodies nor with an HIV-1-positive polyclonal serum in infected astrocytes. Western-blotting assays from the 8E5 T-cell line showed a positive signal with both monoclonal antibodies.

When comparing characteristics of astrocyte infection with non-ADC or ADC viral isolates, we found that this infection was dependent on the isolate. A viral isolate derived from a non-ADC patient (P1) with a cerebral toxoplasmosis was unable to infect astrocytes. The viral strains of the second non-ADC patient (P2) and both ADC patients (P3, P4) induced a transient infection in astrocytes. It must be mentioned that these three patients had neurologic damage directly associated with HIV-1,

the non-ADC patient having an HIV-1 associated multifocal demyelinating neuropathy. We demonstrated that these primary viral isolates were able to infect astrocytes without production of progeny virions in culture supernatants. Previous *in vitro* studies using HIV-1 laboratory-adapted viruses to infect astrocytes, gave discrepant results. A low-production or latent infection was found and cocultivation assays with uninfected lymphoid cells werre necessary to rescue the latent virus (Chiodi *et al*, 1987, Cheng-Mayer *et al*, 1987, Tornature *et al*, 1991). In contrast, a productive infection was observed 3 days post-infection in primary fetal astrocytes inoculated with HIV-1_{SF2} and HIV-1_{IIIB} strains, but not with HIV-1_{ADA-M} strain (Nath *et al*, 1995). This latter was cultured in primary cells before inoculation as were our plasma viral isolates and the HIV-1_{Ba-L} strain used in this study. Absence of viral DNA 3 days post-infection in HIV-1_{ADA-M} astrocytes could have represented a transient infection rather than an inability to infect astrocytes. In our case, we observed that HIV-1 infection of astrocytes was transient and viral DNA was not detected for more than 3 days. Several studies have already shown an abortive infection in astrocytes (Kunsch *et al*, 1989, Keys *et al*, 1991, Shahabuddin *et al*, 1996).

In our study, HIV-1 isolates derived from both ADC patients, presented a similar mRNA pattern in astrocytes, even though they displayed the subtype B and D genotypes in addition to the NSI and SI phenotypes, respectively. Rev and nef but not tat transcripts were detected, and unspliced mRNAs were equally expressed. The non-ADC P2 viral isolate induced the expression of nef but not rev and tat transcripts and the quantity of synthesized unspliced mRNAs was twenty times greater than for the ADC isolates. Our data suggested that infection of astrocytes with HIV-1 isolates followed the same kinetics as T cells or macrophages during the first few hours post-infection (Guatelli *et al*, 1990, Munis *et al*, 1992). Nevertheless, we observed an abortive HIV-1 infection in astrocytes but the mechanisms of this restriction were not resolved.

In fact, nef transcripts were present in HIV-1 infected astrocytes, whatever the viral isolate inoculated. But, we could not detect an accumulation of Nef protein in these cells with our methods. These results confirm those of Wiley *et al* (1996), i.e. nef mRNAs are not preferentially expressed in the brain of ADC patients. In contrast, previous *in vitro* (Kohleisen *et al*,. 1992, Tornatore *et al*, 1994a) and *in vivo* (Tornatore *et al*, 1994b, Saito *et al*, 1994, Ranki *et al*, 1995) studies identified a mechanism of restricted infection due to the accumulation of Nef proteins in astrocytes with or without a very low expression of structural proteins. Alternatively, HIV-1 infection of astrocytes could be restricted to a single cycle in our experiments. The low synthesis of unspliced mRNAs in these cells could prevent the formation or progeny virions. Cellular and/or host factors could interpose in the viral replication cycle and prevent the spread of infection. In the same way, Shahabuddin *et al* (1996) found that restricted astrocyte infection was due to a defective processing of gp160 leading to an absence of gp120 in progeny virus. Lastly, the high replication capacity of normal and tumoral astrocytes in culture could dilute infected cells and contribute to extinguish infection. Cellular cloning or the use of irradiated astrocytes could be helpful tools to study the mechanisms of astrocyte HIV-1 infection.

The impact of transient viral expression in HIV-1 *in vitro* infected astrocytes on HIV-related neurologic damage is unresolved. The different transcriptional patterns found in astrocytes inoculated with ADC and non-ADC isolates should be further investigated in order to better understand the role of these cells in ADC.

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