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Isolation and long-term culture of primary ocular human immunodeficiency virus type 1 isolates in primary astrocytes

M Canki[1], MJ Potash[1], G Bentsman[1], W Chao[1], T Flynn[2], M Heinemann[3], H Gelbard[4] and DJ Volsky[1]

[1]Molecular Virology Laboratory, St. Luke's/Roosevelt Hospital Center and College of Physicians and Surgeons, Columbia University, 432 West 58th St. New York, NY, 10019 USA; [2]Department of Ophthalmology, St, Luke's/Roosevelt Hospital Center, 1000 Tenth Avenue, New York, NY 10019, USA; [3]Ophthalmology Service, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA; [4]Division of Pediatric Neurology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

Vitreous specimens from 14 HIV-1 infected persons undergoing medically indicated vitrectomy were assayed for the presence of infectious HIV-1 and viral tropism. Human primary fetal astrocytes, adult lymphocytes, or macrophages were exposed to vitreous in culture and cells were then assayed for HIV-1 DNA by polymerase chain reaction amplification. We found that 11 of 14 patients tested carried ocular HIV-1 which replicated in one or more primary cell types; of the 13 vitreous samples tested in astrocytes, eight contained transmissible HIV-1. The three patients with no culturable ocular virus were in antiviral therapy at the time of vitrectomy. Comparison of envelope V3 sequences from astrocytes infected in culture to that in uncultured blood cells revealed 21% sequence divergence indicating that ocular HIV-1 transmitted to astrocytes was not recently derived from virus present in the blood. Two ocular samples transmissible to astrocytes were tested further and found capable of sustained replication by serial passage to uninfected astrocytes. However, the viral structural proteins produced by infected astrocytes were abnormal, p24 was absent and higher molecular weight Gag proteins were present. We conclude that the eye is a central nervous system compartment which frequently contains HIV-1 capable of replication in human astrocytes.

Keywords: AIDS dementia; neurotropism; neurovirulence; neuropathology; HIV-1 envelope

Introduction

In the course of infection by HIV-1, approximately 75% of patients develop structural and functional alterations of the nervous system (Masliah *et al*, 1994) resulting in a broad spectrum of clinical presentations in HIV-1 infected persons including the AIDS dementia complex (Price *et al*, 1988); HIV-1 encephalitis (Budka *et al*, 1987); and HIV-1 leukoencephalopathy (Smith *et al*, 1990). It is generally accepted that, at least in children, 'progressive encephalopathy' is directly related to HIV-1 infection of the brain (Belman *et al*, 1994). However, how HIV-1 infection in the brain causes neuropathology is a matter of considerable debate. Replicating HIV-1 can be identified readily in macrophages and microglia in the brain (Sharer *et al*, 1985; Stoler *et al*, 1986; Wiley *et al*, 1986). Cytokines and toxic molecules released from infected cells may be one cause of neuronal damage (Lipton and Gendelman, 1995). On the other hand, an increasing number of reports suggest that HIV-1 can infect astrocytes (Ranki *et al*, 1995; Saito *et al*, 1994; Tornatore *et al*, 1994; Takahashi *et al*, 1996) and neurons (Nuovo *et al*, 1994) *in vivo*. Although virus replication in these cells is restricted and noncytopathic (Nuovo *et al*, 1994; Ranki *et al*, 1995; Saito *et al*, 1994; Tornatore *et al*, 1994), direct infection of neural cells can lead to neural tissue damage by altering cellular gene expression and function (Volsky *et al*, 1992). HIV-1 quasispecies present in different tissues in the body differ from each other in their genetic and functional characteristics (Wolfs *et al*, 1993) and it is important to extend such analyses to

Correspondence: DJ Volsky
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primary HIV-1 strains from the central nervous system (Chiodi *et al*, 1992; Korber *et al*, 1994). To probe a new biopsy source of neural HIV-1 we have obtained vitreous samples for virological studies from AIDS patients undergoing retinal surgery. We found that 11 of 14 vitreous samples contained HIV-1 capable of replication in primary human lymphocytes, macrophages, or fetal astrocytes, and that some infected astrocytes were able to transmit HIV-1 to uninfected astrocytes over a period of months. We conclude that the eye is an accessible compartment suitable for isolation and characterization of neural tissue derived HIV-1.

Vitreous was collected from AIDS patients undergoing vitrectomy either to repair complicated retinal detachments due to concurrent herpes virus infections of the retina or to place Ganciclovir implants. A standard two or three port vitrectomy technique was used (Michels *et al*, 1990). The vitrector was connected to a sterile syringe to aspirate a core vitreous specimen of approximately 0.5–1.0 ml, some of which was used for inoculation of cultures. In most of the cultures described here, vitreous was used within 24 h of collection. Blood was collected from HIV-1 negative donors by venipuncture, peripheral blood lymphocytes (PBL) were isolated by Ficoll-Hypague density sedimentation and monocytes were isolated by centrifugal elutriation. HIV-1 negative fetal astrocytes were prepared from second trimester human fetal brains obtained from elective abortions in full compliance with NIH guidelines. In brief, brain tissue composed of telencephalon with both cortical and ventricular surfaces was dissected and mechanically dissociated by teasing through a Nitex bag and then by filtration through 230 μm and 140 μm sieves and adherence one to several days of culutre in the absence of growth or differentiation factors. This culture system does not support maintenance of microglial cells and contamination with such cells has been shown to be negligible (Nottet *et al*, 1995). Cultures were verified as greater than 95% astrocytic by GFAP staining before infection. PBL were cultured in RPMI supplemented with 5 μg per ml phytohemagglutinin, 10 units per ml interleukin 2, and 10% fetal bovine serum (FBS); monocytes were induced to differentiate and were maintained in DMEM supplemented with 10% giant cell tumor conditioned medium and 10% human serum; astrocytes were cultured in DMEM supplemented with 10% FBS. Two to five million HIV-1 negative PBL, macrophages, or fetal astrocytes were exposed to 50–100 μl vitreous diluted in the appropriate medium lacking serum for 1 h at 37°C. Cells were then washed extensively and were cultured for 1–4 weeks. Uninfected cells were cultured in parallel as negative controls. In some cases as noted in the text, astrocytes were cocultured with uninfected astrocytes at a 1:5 ratio. Cells were harvested after a 2–3 weeks culture period for assay of viral DNA or viral proteins by Western blot analysis.

The presence of HIV-1 DNA synthesized in cultures exposed to vitreous was determined by polymerase chain reaction (PCR) amplification of a highly conserved region in the *gag* gene under standard conditions (Inis *et al*, 1990). Briefly, cells were counted and the relative quantity of cellular DNA in lysates was confirmed by PCR amplification of the single copy human gene β globin. To detect HIV-1 DNA, PCR was conducted for 40 cycles of amplification using the primers SK38 and SK39 (Ou *et al*, 1988) generating a 115 bp fragment. PCR products were separated by electrophoresis in agarose and detected by Southern blot hybridization using the ^{32}P-labeled probe SK19 (Ou *et al*, 1988). A standard curve was constructed by amplifying a plasmid carrying an intact HIV-1 genome, pKS242, in a lysate of uninfected astrocytes. Reaction products of 10 000 test or control cells were loaded for electrophoresis.

Vitreous from HIV-1 infected patients contains transmissible HIV-1 which can infect primary astrocytes *in vitro*

Table 1 summarizes patients' age, reporting conditions, CD4 counts at or near the time of vitrectomy, treatment status, and the transmission of HIV-1 from their vitreous to cells in culture. Except for patient 3, all patients were male. Based upon the presence of HIV-1 capable of viral DNA synthesis in one or more primary cell types, vitreous from 11 of 14 (79%) patients evaluated carried infectious virus of which the majority tested (eight of 13) contained virus transmissible to astrocytes. These results confirm our prliminary report based on a small number of vitreous samples (Flynn *et al*, 1995). All donors of vitreous from which no virus was transmitted were under antiretroviral therapy at the time of donation. The absence of virus in vitreous from these individuals is likely to reflect the overall drop in viral burden with treatment. Assay of HIV-1 DNA in vitreous itself was negative, suggesting that HIV-1 infected cells were not present in vitreous (not shown). Viral DNA burdens were small, 10–50 copies per 10 000 cells, indicating that the titers of HIV-1 in vitreous were low. Consistent with our previous report (Flynn *et al*, 1995), short term cultures inoculated with vitreous expressed no extracellular p24, regardless of their expression of HIV-1 DNA. Taken together, these findings indicate that the eye can serve as an accessible neural compartment in living patients for frequent detection of HIV-1 and establish the first panel of HIV-1 isolates from central nervous system biopsies shown to replicate in primary astrocytes.

Table 1 Clinical and laboratory data for selected vitreous donors

Patient	Age (years)	Ocular disorder	CD4 positive T cell	Treatment anti-HIV-1	Treatment anti-CMV	Ocular HIV-1 isolation Ast	Ocular HIV-1 isolation PBL	Ocular HIV-1 isolation MDM
2	56	CMV-related	<20	AZT	I.V. GCV	–	–	–
3	45	PORN-related	<20	None	I.V. GCV	+	+	ND
4	38	CMV-related	<20	None	I.V. GCV	–	+	–
5	44	CMV-related	<20	None	I.V. GCV	+	+	+
6	40	GCV-related	<20	None	I.V. GCV	+	+	ND
7	33	CMV-related	<20	AZT	I.V. GCV	ND	+	+
9	30	GCV implant	>100	D4T	None	–	+	–
10	33	GCV implant	NR	D4T	I.V. GCV I.V. FOS PO ACT	–	–	–
11	31	GCV implant	NR	3TC	I.V. GCV I.V. FOS PO ACV	–	–	–
12	40	GCV implant	NR	AZT	I.V. GCV I.V. FOS	+	ND	ND
13	58	GCV implant	<20	AZT	I.V. FOS	+	ND	ND
15	42	GCV implant	<20	None	I.V. GCV	+	ND	ND
16	30	GCV implant	<20	None	I.V. GCV	+	ND	ND
17	65	GCV implant	<20	D4T	None	+	ND	ND

Abbreviations: ACV, acyclovir; AST, astrocytes; AZT, 3′-azido-3′-deoxythymidine; D4T, 2′, 3′-didehydro, 3′-dideoxythymidine; FOS, foscarnet; GCV, ganciclovir; MDM, monocyte derived macrophages; ND, not determined; NR, not reported; PORN, progressive outer retinal necrosis; 3TC, 2′-deoxy, 3′-thiacytidine.

HIV-1 Env sequence of vitreous-derived virus differs from the sequence of virus in lymphocytes

The ability to amplify HIV-1 DNA in cells infected by vitreous virus permitted comparison of the virus transmitted to astrocytes and the virus resident in PBL of the donor at the time of vitrectomy by *env* sequence analysis. Nested PCR was conducted on extracts of astrocytes exposed to vitreous from patient 15 or on extracts of patient 15 PBL using sense primer (5′-CCGGATCCTACAAATGTCAGCA-CAGT-3′) and antisense primer (5′-GGGAATT-CTCTTTGCCTTGGTGGGTG-3′) which amplify the region spanning nt 6940–7730 (HXB-2 numbering) (Myers *et al*, 1992) followed by amplification using the primers BRUV5 and BRUV3 (Wain-Hobson *et al*., 1991) to amplify the segment nt 7012–7346 containing the envelope V3 region. The bulk PCR products were sequenced and the deduced amino acid sequences around the V3 region were aligned (Figure 2). There is 79% homology of HIV-1 envelope amino acid sequences from astrocytes infected in culture and those from uncultured PBL, compared to 75% or 76% homology of each of these sequences to the North American consensus sequence (Myers *et al*, 1992). These findings suggest that the vitreous HIV-1 capable of replication in astrocytes was not recently derived from blood cells. It has been noted that intrapatient variation in the hypervariable regions of HIV-1 gp120 envelope sequence can equal or exceed interpatient variation (Martins *et al*, 1992).

It is noteworthy that the HIV-1 transmitted to astrocytes contained a highly positively charged V3 region, different from previously described brain-derived HIV-1 (Donaldson *et al*, 1994; Li *et al*, 1991; Monken *et al*, 1995; Westervelt *et al*., 1992). The predominant HIV-1 strains in the brain are macrophase-tropic and are produced by macrophages and microglial cells and not astrocytes as we examine here. A recent study of HIV-1 replication in brain capillary endothelial cells reported that tropism to these cells assorts neither with tropism to macrophages nor tropism to transformed T cells (Moses *et al*, 1996). These results suggest that HIV-1 transmissible to CD4 negative cells, like that we identify here in vitreous, may constitute a novel class of virus.

Persistent culture and unusual biology of ocular HIV-1 in primary astrocytes

Previous studies have demonstrated that primary astrocytes are susceptible to infection with a laboratory strain of HIV-1 (Cheng-Mayer *et al*, 1987; Fiala *et al*, 1996; Tornatore *et al*, 1991; Wigdahl *et al*, 1987), but viral replication was transient, followed by the establishment of a semi-latent state characterized by the expression of mRNAs encoding Rev and Nef (Tornatore *et al*, 1994). To determine whether ocular HIV-1 can establish a viral reservoir in neural tissue, we used astrocytes exposed to vitreous from patients 12 and 15 as a source of virus and cocultured them with uninfected astrocytes at a 1:5 ratio (Figure 1). As shown in Figure 1, viral DNA was detected after five passages of astrocytes originally infected by vitreous 12 or by vitreous 15. We have continued passage of the patient 15 isolate for 11 passages of

more than 6 months of continuous culture, with DNA burdens within four weeks of co-culture reaching 200–500 copies per 10 000 cells. These findings indicate that ocular HIV-1 is capable of sustained replication and spread in astrocytes. Viral p24 expression in ocular HIV-1 infected astrocytes was not detectable by commercial p24 Elisa. To determine if this was representative of viral structural proteins generally, we performed Western blot analysis using anti-Gag monoclonal antibody (Simm *et al*, 1995). We tested extracts of astrocytes which had been serially cocultured with astrocytes exposed to vitreous from Patient 15 (Figure 3). We were surprised to find anti-Gag reactive proteins of about 41 kDa and 160 kDa, which correspond to an immature Gag polyprotein and unprocessed Gag-Pol, but no mature p24. This finding is consistent with previous reports of an HIV-1 life cycle in astrocytes different from that in lymphocytes (Saito *et al*, 1994; Tornatore *et al*, 1994; Volksy *et al*, 1992).

Summary and implications

We have found that the majority of AIDS patients requiring vitrectomy tested in this study carry transmissible HIV-1 in their vitreous. Moreover, ocular HIV-1 was infectious to human astrocytes, one of the cell types posited to play a role in the development of AIDS dementia (Saito *et al*, 1994; Tornatore *et al*, 1994; Volsky *et al*, 1992 Takahashi *et al*, 1996). To our knowledge, ours is the first system for transmission of primary HIV-1 strains from neural tissue to primary neural cells.

Four conclusions can be drawn from our findings. First, as a group, patients under antiretroviral therapy contained less transmissible HIV-1 in vitreous than those without antiretrovirals. We interpret this finding to indicate that overall viral burdens in such patients are reduced and that under such circumstances entry of virus into the nervous system may be inefficient. Second, in comparison of sequences from bulk PCR products, HIV-1 transmissible to astrocytes was considerably different in Env sequence than that present in circulating lymphocytes. This suggests that this HIV-1 species had not recently left the circulation to enter the vitreous compartment and is not due to blood contamination. Third, HIV-1 from vitreous was able to spread in

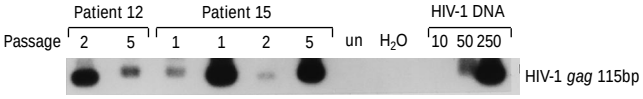

Figure 1 Serial passage of ocular HIV-1 in astrocytes as determined by HIV-1 DNA detection. Astrocytes exposed to vitreous from Patient 12 or 15 as indicated were cocultured with uninfected astrocytes and were subjected to PCR amplification of HIV-1 *gag* DNA as described in Methods. Cells from day 25 of Passage 2 and day 15 of Passage 5 of Patient 12 cultures were analysed; cells from day 7 and 17 of Passage 1, day 25 of Passage 2, and day 19 of Passage 5 of Patient 15 cultures were analysed. Un indicates amplification of DNA from uninfected astrocytes and H_2O indicates amplification with no cell lysate. 10, 50, or 250 copies of HIV-1 DNA in lysates of uninfected astrocytes were amplified in parallel as standards.

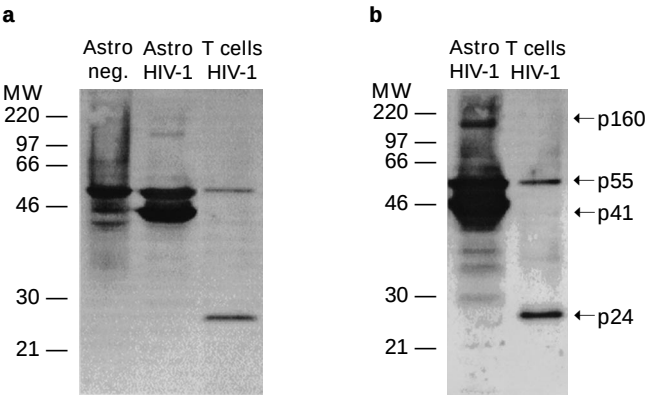

Figure 3 HIV-1 Gag proteins produced by astrocytes exposed to Patient 15 vitreous. Uninfected astrocytes (Astro neg.), astrocytes exposed to Patient 15 vitreous (Astro HIV-1) or CEM T leukemia cells infected with HIV-1/N1T (T cell HIV-1) were lysed and electrophoresed in SDS-polyacrylamide under standard conditions (Ausubel *et al*, 1987), the proteins were elctroblotted onto Nitrose cellulose and stained with monoclonal anti-Gag antibody AG 3.0 (Simm *et al*, 1995). (**b**) is a longer exposure of selected lanes from (**a**) to permit better visualization of atypical Gag-specific proteins present in ocular HIV-1 infected astrocytes. Typical Gag-specific proteins recognized by AG 3.0 antibody are marked on the right of (**b**).

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                    10        20        30        40        50        60        70        80        90
                    *         *         *         *         *         *         *         *         *
consensus B         AEEEVVIRSENFTDNAKTIIVQLNESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNNTLKQIVKKLREQFGN-KTI
15 vitreous/astroyctes  .........................V...........RRLS........ARRN.....................Q.V.I....K.R.-...
15 PBL              .........D..S........H..A..............G.N........A..D.............KR...E..E.V.I...Q....N...
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Figure 2 HIV-1 Envelope region sequences from astrocytes exposed to Patient 15 vitreous or from Patient 15 PBL. PCR was performed as described in the text, DNA was cloned into the pTA cloning vector, isolated under standard conditions (Ausubel *et al*, 1987) and sequenced at the Columbia Comprehensive Cancer Core Facility. Amino acid sequences were deduced from the DNA sequences and were aligned using the Align program versus the North American HIV-1 Env consensus sequence (Myers *et al*, 1992).

culture to astrocytes over serial passages. Viral DNA burdens increased 10-fold during passage and since the infected cell population was diluted fivefold during co-culture, at least a 50-fold increase in viral titer occurred throughout each passage in culture, indicating that ocular HIV-1 is infectious and capable of sustained replication in astrocytes. Fourth, viral proteins produced by astrocytes infected by ocular HIV-1 were abnormal, Gag proteins were made but inefficiently processed, offering a possible explanation for the inefficient transmission of this virus in culture and suggesting that the overall HIV-1 life cycle is different in astrocytes than in lymphocytes (Cheng-Mayer *et al*, 1987; Nath *et al*, 1995; Saito *et al*, 1994; Tornatore *et al*, 1991, 1994; Volsky *et al*, 1992; Wigdahl *et al*, 1987). This infection system will enable us to test some of the properties of astroyctes infected by an HIV-1 strain from the central nervous system which may clarify their role in AIDS neuropathogenesis.

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