

Isolated Human Astrocytes Are Not Susceptible to Infection by M- and T-Tropic HIV-1 Strains Despite Functional Expression of the Chemokine Receptors CCR5 and CXCR4

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KEY WORDS chemokine receptors; CD4; HIV; astrocytes; microglial cells; development

ABSTRACT Within the brain, HIV-1 targets the microglia and astrocytes. Previous studies have reported that viral entry into astrocytes is independent of CD4, in contrast to microglia. We aimed to determine whether chemokine receptors play a role in mediating CD4-independent HIV-1 entry into astrocytes. We found that embryonic astrocytes and microglial cells express CCR5, CCR3, and CXCR4 transcripts. Intracellular calcium levels in astrocytes were found to increase following application of RANTES, MIP-1 β (CCR5-agonist), SDF-1 α (CXCR4-agonist), but not eotaxin (CCR3-agonist). In microglial cells, eotaxin was also able to modulate internal calcium homeostasis. CD4 was not present at the cell surface of purified astrocytes but CD4 mRNA could be detected by RT-PCR. Neither HIV-1₉₅₃₃ (R5 isolate) nor HIV-1_{LAI} (X4 isolate) penetrated into purified astrocytes. In contrast, mixed CNS cell cultures were infected by HIV-1₉₅₃₃ and this was inhibited by anti-CD4 mAb in 4/4 tested cultures and by anti-CCR5 mAb in 2/4. Thus, the HIV-1 R5 strain requires CD4 to penetrate into brain cells, suggesting that CCR5 cannot be used as the primary receptor for M-tropic HIV-1 strains in astrocytes. Moreover, inconstant inhibition of HIV-1 entry by anti-CCR5 mAb supports the existence of alternative coreceptors for penetration of M-tropic isolates into brain cells. *GLIA* 34:165–177, 2001. © 2001 Wiley-Liss, Inc.

INTRODUCTION

The cellular tropism displayed by different human immunodeficiency virus type 1 (HIV-1) isolates is related to their use of specific chemokine receptors that mediate HIV-1 entry in conjunction with CD4. Virus strains that infect primary T cells and transformed T-cell lines (T-tropic strains) use the α -chemokine receptor CXCR4 (X4 strains), while those that replicate in primary T cells and macrophages (M-tropic strains) typically use the β -chemokine receptor CCR5 (R5 strains) (Berger et al., 1998; Miller and Meucci, 1999). Other chemokine receptors such as CCR2b, CCR3, CCR8, CCR9, CX₃CR1, and APJ in addition to some orphan receptor such as GPR1, GPR15, and STRL33 have been shown to bind HIV and SIV envelopes (for review, see Berger et al., 1999). In vivo,

the contribution of these alternative receptors to infection and pathogenesis remains obscure. HIV-1 invades the

Abbreviations used: AIDS, acquired immunodeficiency syndrome; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde phosphodehydrogenase; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; MDM, monocyte-derived macrophage; MIP, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PTX, pertussis toxin; RANTES, regulated on activation normal T expressed and secreted; SDF, stromal cell-derived factor; SIV, simian immunodeficiency virus.

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central nervous system (CNS) early after systemic infection and causes severe neurological complications that can be observed in the late stages of acquired immunodeficiency syndrome (AIDS). Cells that are productively infected are resident activated microglia and macrophages that have transmigrated through the blood-brain barrier (Watkins et al., 1990; Lee et al., 1993; Tardieu, 1999). In astrocytes, HIV-1 infection is restricted to the expression of early regulatory gene products (e.g., Nef and Rev), suggesting that HIV-1 establishes a nonproductive infection in these cells (Saito et al., 1994; Tornatore et al., 1994; Ranki et al., 1995). Such an infection might contribute to the persistence of the virus in the brain. In vitro studies have reported that, in contrast to microglia (Jordan et al., 1991), HIV-1 infection of astrocytes is CD4-independent (Harouse et al., 1989; Tornatore et al., 1991). Whether chemokine receptors play a role in mediating CD4-independent entry into astrocytes is still an open question.

The expression of chemokine receptors in brain has been widely investigated but has produced controversial results in astrocytes. Immunocytochemical studies on healthy human brain or brain with HIV-encephalitis have revealed the presence of CXCR4, CCR5, and CCR3 in astrocytes and microglial cells (Rottman et al., 1997; Lavi et al., 1997; Vallat et al., 1998; Sanders et al., 1998; Ghorpade et al., 1998). Furthermore, functional expression of CXCR4 and CCR5, but not CCR3, has been demonstrated on cultured fetal astrocytes (Klein et al., 1999; Andjelkovic et al., 1999) and adult microglia (Albright et al., 1999) by microphotometry. In contrast, Sabri et al. (1999) failed to detect coreceptor at the cell surface of cultured fetal astrocytes, although mRNAs of CXCR4 and CCR5 in addition to transcripts of the orphan receptors STRL33 and APJ were present. These observations suggest a heterogeneous expression of chemokine receptors among subpopulations of astrocytes or variations dependent on the stage of development.

HIV entry and the role of chemokine receptors as coreceptor have been investigated mainly in microglia among CNS cells. T-tropic viruses that use CXCR4 penetrate them relatively inefficiently (He et al., 1997; Shieh et al., 1998). The penetration of brain-derived HIV isolates can be mediated by CCR3 and CCR5, together with CD4 (He et al., 1997; Shieh et al., 1998; Albright et al., 1999) but Ghorpade et al. (1998) failed to inhibit viral entry of both M-tropic and brain-derived strains by anti-CCR5 and anti-CCR3 mAbs in microglia. This suggests that microglia express additional chemokine receptors that function as HIV coreceptor. Recently, Albright et al. (1999) reported that some HIV-1 strains isolated from the CNS can use CCR8 in transfected cell lines and Jinno et al. (1998) showed expression of CCR8 in a glioma cell line and its involvement in viral entry.

To further study the chemokine receptors used as cofactors for HIV-1 entry into human astrocytes, we investigated the functional expression of CXCR4, CCR5 and CCR3 in highly purified embryonic astrocyte cultures. The results were compared with those obtained from microglial cultures tested in parallel. Viral

penetration into astrocytes was quantified by measuring early steps in HIV reverse transcription by semi-quantitative PCR and was compared with infection of mixed CNS cells or MDM with the same HIV-1 isolates.

MATERIALS AND METHODS

Cell Preparation And Culture

Primary CNS cell cultures

Primary mixed CNS cell cultures were prepared as previously described (Janabi et al., 1996) from prosencephalon and spinal cord of 8–10-week-old human embryos obtained after elective abortion (performed in full compliance with both French National Ethics and local ethics committee guidelines). Briefly, brain tissue was dissected, trypsinized and the cell suspension was distributed at a density of 10^6 cells/35-mm culture well coated with collagen in MCDB medium (Seromed, France), supplemented with 5% fetal calf serum (FCS), antibiotics (10^5 U/L penicillin, 0.1 g/L streptomycin), and 2 mM glutamine. Cultures were placed in a 10% CO₂-humidified incubator at 37°C. The medium was removed 48 h after plating and changed by one-half every 3–4 days. Cultures consisted of nerve cell adhesion molecule- and neurofilament-positive neuronal clusters lying on a monocellular layer containing 50–70% glial fibrillary acidic protein (GFAP)-positive astrocytes and 30–50% CD68/KiM-7-positive microglial cells as described (Janabi et al., 1998).

Purified cultures of microglia or astrocytes

Ten to 15 days after plating, circular shaking of primary CNS cell cultures allowed removal of microglial cells, which were transferred and selected by adherence. These cultures were subsequently grown for 3 weeks to 2 months and contained more than 98% of CD68/KiM-7-positive cells (Janabi et al., 1996) (Fig. 1). The remaining adherent cells were trypsinized, seeded into 175-cm² flask and passaged 3–4 times to obtain purified cultures of astrocytes. Before each trypsinization, astrocyte cultures were submitted to an additional circular shaking to avoid microglial cell contamination. Three-week-old (passage 3) astrocyte cultures were >99% pure, as determined by GFAP immunostaining, and contained no CD68/KiM-7-positive cells (Janabi et al., 1996) (Fig. 1).

Preparation of human macrophages

Human PBMC from healthy HIV-seronegative donors were isolated by Ficoll-Hypaque gradient centrifugation. Monocytes were selected by a 2 hour adherence in Lab-tek tissue-culture chamber/slides (OSI, France) at 37°C in a 10% CO₂ atmosphere. Monocytes differentiated into macrophages for 15 days in culture

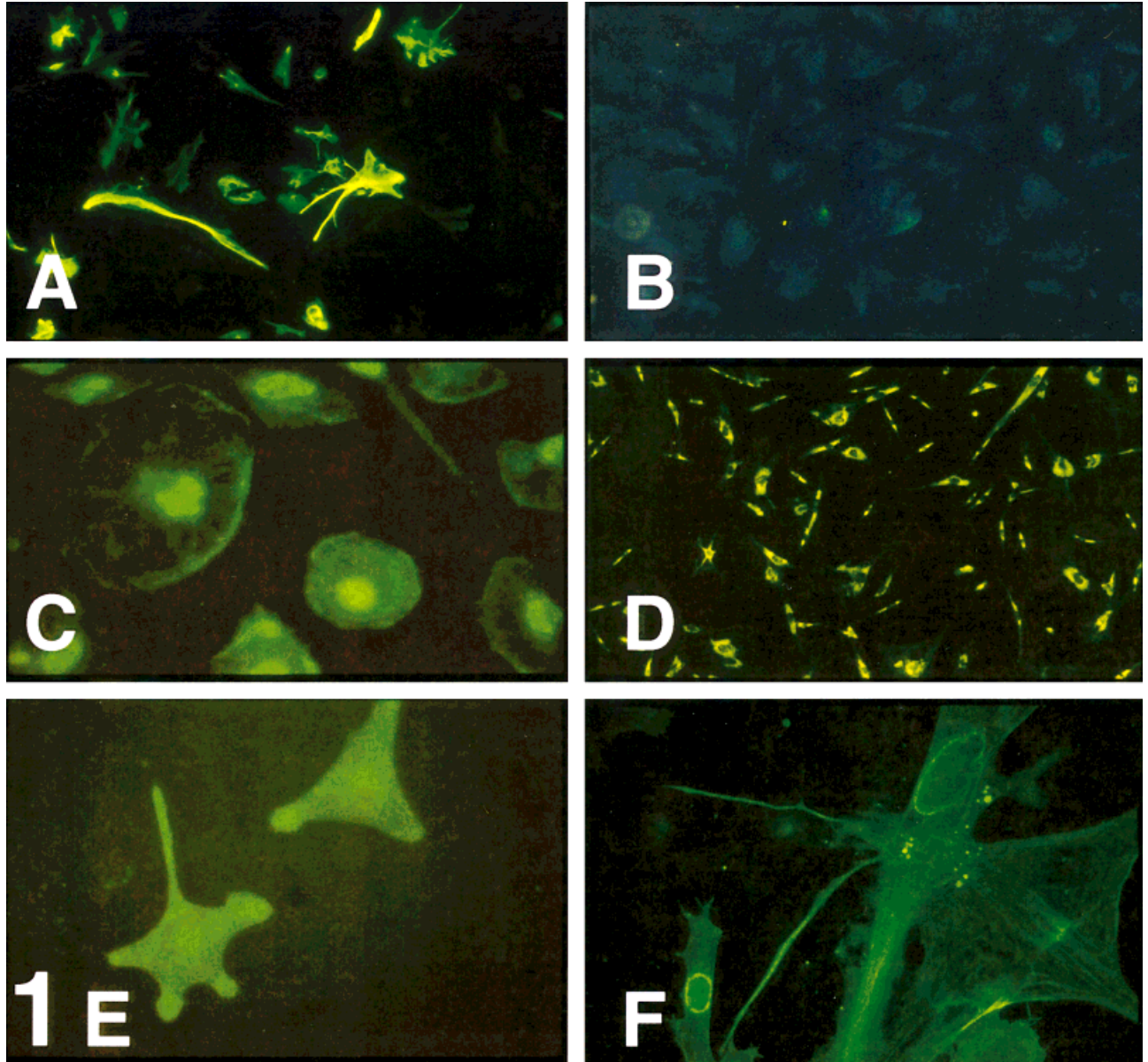


Fig. 1. Specific staining of human embryonic astrocytes and microglia and immunofluorescence labeling of CXCR4, CCR5, and CCR3 in astrocytes. Astrocytes (passage 4–5) or microglia (passage 2–3) were cultured in Lab-tek tissue-culture chamber/slides at a density of 30,000 cells/well and fixed with methanol. Astrocyte cultures were >99% pure, as determined by glial fibrillary acidic protein (GFAP) immunofluorescence staining (A) and contained no CD68/KiM-7-positive cells (B). Conversely, microglial cultures contained more than

98% of CD68/KiM-7-positive cells (D). Astrocytes were stained with the (C) 12G5 anti-CXCR4 mAb, (E) MAB181 anti-CCR5 mAb, (F) 7B11 anti-CCR3 mAb, and binding was demonstrated by biotin-conjugated secondary antibody followed by FITC-conjugated avidin. The results are representative of three independent experiments performed with three separate embryonic brain tissue specimens. A, B, D: x100 and C, E, F: x400.

in RPMI-1640 medium (Seromed) containing 10% FCS, antibiotics and glutamine. Monocyte-derived macrophage (MDM) cultures contained more than 95% of CD68/KiM-7-positive cells.

Materials

Recombinant human β chemokines MIP-1 α , MIP-1 β , RANTES, eotaxin and the α chemokine SDF-1 α were

purchased from R&D Systems (Oxon, UK) and used at 100 ng/ml for photometric experiments. Bordetella pertussis toxin (PTX; 250 ng/ml) was obtained from Sigma (St. Quentin Fallavier, France). Monoclonal Ab to CCR5 (2D7; PharMingen, San Diego, CA; Wu et al., 1997), CCR3 (7B11; AIDS Research & Reference Reagent Program, National Institutes of Health [NIH], Rockville, MD), and CXCR4 (12G5; R&D Systems; Endres et al., 1996) were used to inhibit HIV-1 infection. HIV-blocking anti-CD4 mAb (Leu3a) was purchased

TABLE 1. Nucleotide primers used for polymerase chain reaction

Amplification product	Primer	Sequence	PCR product (bp)
CXCR4	Sense (5')	ACTACACCGAGGAAATGGGCTC	239
	Antisense (3')	TGATGACAAAGAGGAGGTCGGC	
CCR5	Sense (5')	GCTGTGTTTGCCTCTCTCCCA	235
	Antisense (3')	GCCCTGTGCCTCTTCTTCT	
CCR3	Sense (5')	GGACTGTCACTTTTGGTGTCAATCA	211
	Antisense (3')	TAACGAGCAGAGGGAGAACGAG	
CD4	Sense (5')	TAGCCCTCAGTGCAATGTAGG	277
	Antisense (3')	TGCCCCGTCAGCTTCAACTG	
HIV-1 R/U5	Sense (5')	biotin-GGGAACCCACTGCTTAAGCCT	99
	Antisense (3')	TGAGGGATCTCTAGTTACCAG	
	probe	GAAGCACTCAAGGCAAGC-digoxigenin	
GAPDH	Sense (5')	biotin-GGTGAAGGTCGGAGTCAACGGA	242
	Antisense (3')	GAGGGATCTCGCTCCTGGAAGA	
	probe	AAAGCAGCCCTGGTGACC-digoxigenin	

from Becton Dickinson (San Jose, CA). Anti-CD4 and anti-CCR5 mAb were dialyzed against PBS overnight at 4°C to remove sodium azide. Immunolabeling experiments were performed with anti-CCR5 mAb MAB181 obtained from R&D System.

HIV-1 Strains

The HIV-1 T-tropic isolate LAI, passed through a permanent T-cell line and known to use CXCR4 (Simmons et al., 1996), was kindly provided by Dr. Barré-Sinoussi (Institut Pasteur, Paris, France). The laboratory-adapted M-tropic strain HIV-1₉₅₃₃ (a gift of Dr. Molina and Dr. Sinet) was grown in MDM. Before use, virus supernatants were treated with 50 U/ml RNase-free DNase I (Roche Diagnostic, Germany) for 30 min at room temperature to remove contaminant viral DNA. The HIV-1₉₅₃₃ strain was characterized regarding the coreceptor usage by infection of MDM in the presence of various inhibitors of chemokine receptors. Positive entry of the virus into MDM was confirmed by comparison of viral DNA levels in cells after exposure to infectious HIV-1₉₅₃₃ or to heat-inactivated HIV-1₉₅₃₃ (2 h pi: 0.8 ± 0.08 vs 0.01 ± 0.01 HIV DNA units; infectious vs heat-inactivated virus; $n = 2$). HIV-1₉₅₃₃ infection of macrophages was inhibited by anti-CCR5 (2D7; 0.2 µg/ml; 8 h postinfection: 2 ± 0.3 vs 5.8 ± 0.8 HIV DNA units, 2D7-treated vs control; $n = 2$) and anti-CD4 (Leu3a; 12.5 µg/ml; 8 h pi: 1.7 ± 0.8 vs 5.8 ± 0.8 HIV DNA units, Leu3a-treated vs control; $n = 2$) mAb, but neither by eotaxin (100 ng/ml; 8 h pi: 12.4 ± 4 vs 5.8 ± 0.8 HIV DNA units, eotaxin-treated vs control; $n = 2$) nor by anti-CXCR4 mAb (12G5; 20 µg/ml; 8 h pi: 11.2 ± 2 vs 5.8 ± 0.8 HIV DNA units, 12G5-treated vs control; $n = 2$). We considered a significant difference between values of both conditions when one-log-separated. This supports the identification of the M-tropic HIV₉₅₃₃ strain as a R5 isolate.

HIV-1 Infection of Brain Cells

Astrocytes seeded in 12-well plates (5×10^5 cells/well), or primary mixed CNS cells were incubated over-

night (12 h) with viral inoculum (25 ng of p24 gag antigen/well) at 37°C, washed several times and kept in culture until lysis. Cells were also pulsed with heat-inactivated virus (1 h at 56°C) to determine baseline contamination of DNA in the viral stocks. For inhibition experiments, cells were preincubated for 45 min before infection with monoclonal antibodies described above. DNA was extracted using the Qiamp blood kit (Qiagen, Germany) at regular times after viral exposure (12, 18 h) and R/U5-PCR assay was performed to assess HIV penetration.

Semiquantitative PCR of HIV-1 DNA

Semiquantification of DNA was carried out using kinetic polymerase chain reaction–enzyme-linked immunosorbent assay (PCR-ELISA), as reported by Taoufik et al. (1997). The oligonucleotide primer pair specific for the R/U5 region of the LTR was used to assess viral entry (Table 1). This primer pair flanks sequences within the first region of the viral DNA synthesized during reverse transcription, as previously described (Zack et al., 1990). PCR was performed on 10-µl DNA samples. Aliquots of PCR reactions were sampled every three cycles and transferred onto avidin-coated microplates. The 5'-biotinylated PCR products were incubated in microplates and denatured with NaOH. The captured strand was hybridized with a digoxigenin-labeled probe and the bound probe was detected using alkaline phosphatase-coupled anti-digoxigenin antibody. Chemiluminescent substrate for alkaline phosphatase (CSPD, Roche Diagnostic) plus luminescence enhancer (Sapphire; Tropix, Bedford, MA) were added; luminescence was measured using Micro Beta Plus (Wallac, Turku, Finland). The amounts of amplicons quantified by enzyme-linked immunosorbent assay (ELISA) were compared by linear regression with an external scale of DNA at the cycles where signals were in the linear portion of the revelation assay. GAPDH was amplified and quantified for each DNA preparation to correct for variability in DNA recovery. Results were expressed as relative DNA units (calculated as

the ratio of HIV DNA to GAPDH, both in Ag/ μ l) and are presented as the average of duplicate determinations.

Immunostaining

Astrocytes or microglia cultured for 3 weeks to 2 months were seeded in Lab-tek tissue culture chamber/slides at a density of 30,000 cells/well and fixed in either methanol or 2% paraformaldehyde. Monocytes purified from PBMC were differentiated into macrophages for 15 days *in vitro*. Cells were incubated 1.5 hours at room temperature with either anti-GFAP (2 μ g/ml), anti-CD68 (KiM-7; 2 μ g/ml), anti-CXCR4 (12G5; 20 μ g/ml), anti-CCR5 (MAB181; 10 μ g/ml), or anti-CCR3 (7B11; 20 μ g/ml) mAb. Immunostaining was demonstrated by secondary biotin-conjugated antibody and FITC-avidin (Vector, CA) and FITC-labeled cell cultures were analyzed by inverted microscopy (Zeiss, Germany).

RT-PCR Analysis

RNA was isolated from 10^6 astrocytes, microglial cells, or MDM, using the method of Chomczynsky and Sacchi (1987), as modified in the RNAxis™ system (Genaxis, France). Total RNA was treated with 25 U of RNase-free DNase I (Roche Diagnostic) for 60 min at room temperature in the presence of 10 mM $MgCl_2$, with subsequent inactivation at 70°C for 15 min. cDNA synthesis was performed in a 20- μ l volume containing 5 μ l of RNA, 3.2 μ g of random primer p(dN)6, 1.6 μ g of oligo(dT)15 primer, 1 mM (each) dNTP, 50 U of RNase inhibitor, and 20 U of avian myeloblastosis virus-reverse transcriptase (first-strand cDNA synthesis, Roche Diagnostic). PCR amplifications were performed in 50 μ l reactions containing 4 μ l of cDNA, 1 \times PCR buffer, 1.25 U Platinum™ *Taq* DNA Polymerase (Gibco-BRL, France), 0.2 mM of each dNTP (Promega, Madison, WI), 1.5 mM $MgCl_2$, and 0.25 pmol of each primer (Table 1), by using a PTC-100 thermal cycler (MJ Research, Watertown, MA; 10 min at 94°C; 42 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C; and 5 min at 72°C). RT-PCR products were analyzed on a 2% agarose gel in the presence of ethidium bromide. Control cDNA reactions without reverse transcriptase were run in parallel to verify the absence of contamination of genomic DNA.

Calcium Flux Assay

Changes in the intracellular calcium concentrations ($[Ca^{2+}]_i$) were monitored using the fluorescent probe Indo-1 pentaacetoxymethylester (Indo-1 AM, Molecular Probes, Eugene, OR) according to the technique described by Grynkiewicz et al. (1985). Astrocytes or microglia, plated on coverslips, were used for 3 weeks to 2 months. Cells were labeled with 5 μ M Indo-1 for 30

min at 37°C. Medium was then removed, and cells were stimulated with chemokines for 90 s in solution containing 5 mM $CaCl_2$ as previously reported (Lannuzel et al., 1995). Samples were excited at 355 nm with continuous stirring and the Indo-1 fluorescence was measured as a function of time at both 405 nm and 490 nm, using an inverted microscope (Nikon Diaphot, Japan) fitted with epifluorescence ($\times 40$ glycerol immersion fluorescent objective) and equipped for microfluorimetry (PhoCal system, Life Science Resources, Cambridge, UK). Results are expressed as the ratio of values obtained at the two emission wavelengths as a function of time (seconds).

RESULTS

Expression of Functional Chemokine Receptors on Glial Cells

Astrocytes and microglia express CXCR4, CCR5, and CCR3 mRNA

RT-PCR were performed using specific primers for CXCR4, CCR5, and CCR3 on 8–19 purified cultures of astrocytes and microglia obtained from different brain specimens. Total RNA from 10^6 glial cells was treated with DNase before reverse transcription, to eliminate contaminating genomic DNA. CXCR4, CCR5, and CCR3 transcripts were detected in both glial cell types (Fig. 2). The identity of the transcripts was confirmed by DNA sequencing of the PCR products. However, mRNA expression was not constant in the different cultures tested. In astrocytes, RT-PCR were positive in 16/19 cultures for CXCR4, but in only 3/17 for CCR5. Seven out of 9 cultures of microglia were positive for CXCR4 mRNA, and 3 out of 8 for CCR5 mRNA. Finally, CCR3 mRNA was present in 4/16 different astrocyte cultures and in 5/9 for microglia. MDM, isolated from 3 separate adult donors, were used as positive controls for the 3 transcripts (Fig. 2) and invariably expressed the tested messengers, except CCR3 (Ponath et al., 1996).

Detection of CXCR4, CCR5, and CCR3 protein

To establish that astrocytes expressed CXCR4, CCR5, and CCR3 protein, cells in highly purified astrocyte cultures were stained with specific anti-CXCR4 (12G5), anti-CCR5 (MAB181) and anti-CCR3 (7B11) mAb. Figure 1 demonstrated positive immunostaining for CXCR4 (98% labeled astrocytes), for CCR5 (62% labeled astrocytes), and for CCR3 (41% labeled astrocytes). CCR5-, CCR3- and CXCR4-positive astrocytes display typical morphology of astrocytes: either star-shaped (CCR5 and CCR3 staining: Fig. 1E,F) or protoplasmic (CXCR4 staining: Fig. 1C) type. Both morphologies are visible in purified cultures of astrocytes (Fig. 1A).

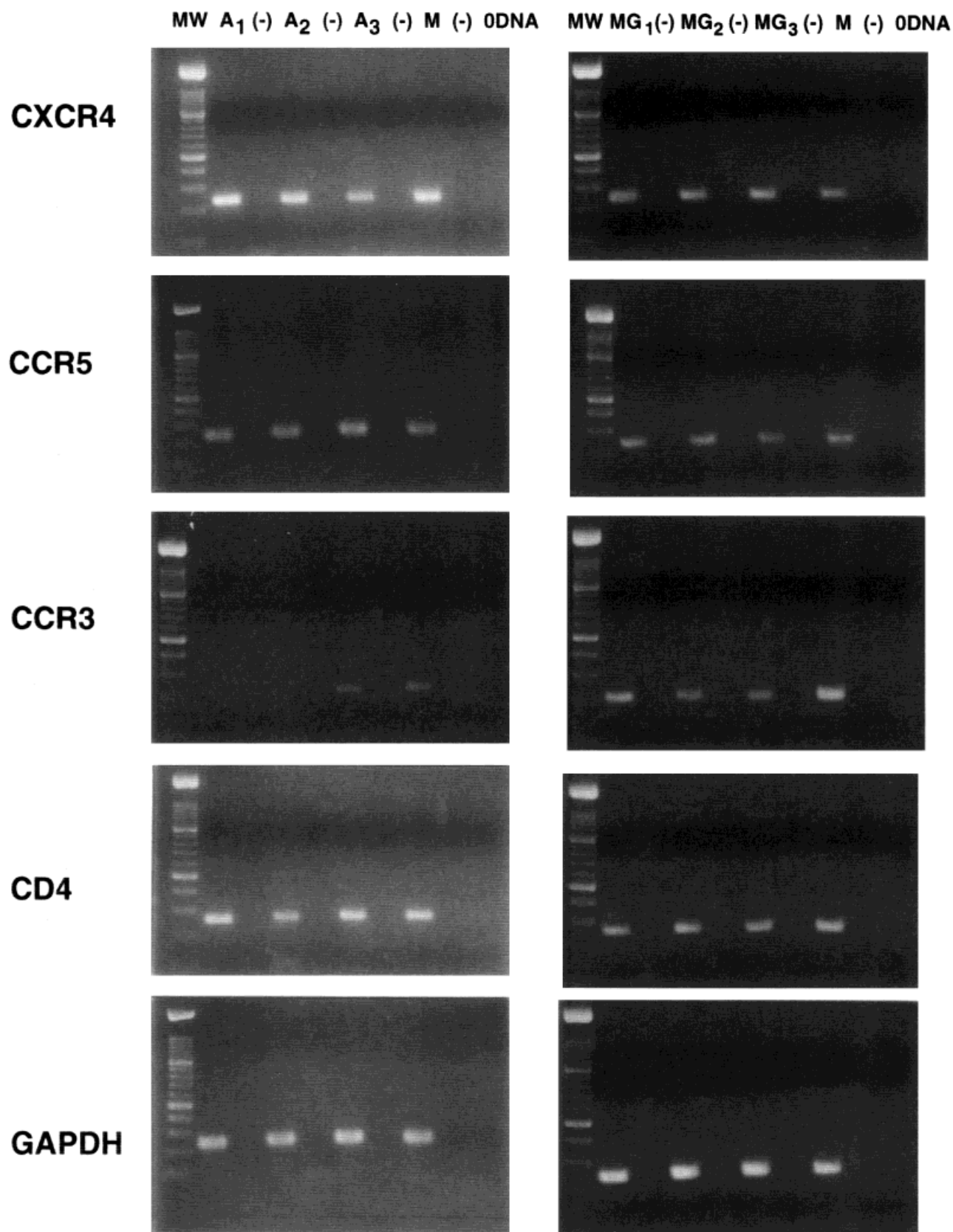


Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) detection of chemokine receptor mRNA in astrocytes and microglia. DNase-treated RNA from the cell types indicated above each lane were used as a template for PCR before (–) and after reverse transcription using primers specific for the indicated chemokine receptors

and CD4. PCR products for CXCR4 (239 bp), CCR5 (235 bp), CCR3 (211 bp), CD4 (277 bp), and GAPDH (242 bp) were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Amplicons from three different astrocyte (A_{1–3}) or microglial (MG_{1–3}) cultures and from MDM (M) are shown.

SDF-1 α , MIP-1 β , and RANTES induce transient elevation of [Ca²⁺]_i in astrocytes and microglia

Chemokines have been shown to carry out their biological activity through induction of calcium flux in leukocytes (Bokoch, 1995). To determine the physiological significance of the presence in glial cells of mRNA encoding the chemokine receptors, we examined the ability of SDF-1 α , the specific ligand for CXCR4, and eotaxin, specific for CCR3 to mobilize calcium in single-cell experiments. RANTES was also used as a broad range agonist for β -chemokine receptors and MIP-1 β as a specific agonist for CCR5. Between 3 to 5 isolated astrocytes or microglial cells were tested per culture for their responses to each chemokine. Astrocytes from 20 cultures prepared from different embryonic brain tissue specimens exhibited an increase in calcium levels when stimulated with 100 ng/ml of SDF-1 α (28% of responding cells; $n = 85$ tested astrocytes), RANTES (36%; $n = 44$) and MIP-1 β (25%; $n = 74$) (Fig. 3A). Detectable calcium mobilization was observed with 25 ng/ml of chemokine. In contrast, eotaxin at a concentration up to 500 ng/ml failed to induce a calcium response in astrocytes ($n = 25$) (Fig. 3A). Ten different purified microglial cultures were tested and increased intracellular calcium concentrations were observed with SDF-1 α (12% of responding cells; $n = 43$), RANTES (10%; $n = 30$), MIP-1 β (18%; $n = 50$) and eotaxin (13%; $n = 16$); each chemokine was tested at 100 ng/ml (Fig. 3A).

Sequential stimulation of astrocytes with agonists that share receptors (MIP-1 α : ligand of CCR1 and CCR5, MIP-1 β : ligand of CCR5 (Raport et al., 1996), and CCR8 (Bernardini et al., 1998) was used to assess cross-desensitization. As shown in Figure 3B, 500 ng/ml of MIP-1 α (a concentration that produced maximal responses) abrogated the response to 100 ng/ml MIP-1 β . After washing, astrocytes were still able to respond to MIP-1 β (Fig. 3B). We also tested the β -chemokine I-309 (100 ng/ml), which specifically binds CCR8 (Tiffany et al., 1997) and failed to trigger a calcium response in glial cells (astrocytes, $n = 7$; microglia, $n = 3$; data not shown).

Chemokines have been shown to bind to 7-transmembrane domain receptors associated with PTX-sensitive G α protein (Wu et al., 1993; Murphy, 1994). [Ca²⁺]_i increases induced by RANTES in astrocytes and microglia were inhibited when cells were pretreated for 2.5 h with 250 ng/ml of PTX (Fig. 4). The same inhibition was observed for MIP-1 β and SDF-1 α (MIP-1 β , $n = 7$; SDF-1 α , $n = 2$; data not shown). These results indicate that the agonists used were acting by recruiting G α protein-coupled receptors (GPCR).

CD4 Expression

We found that astrocytes expressed the messenger for CD4 (Fig. 2). However, no CD4 cell surface expression on astrocytes was detected by fluorocytometry

(method described in Mognetti et al., 2000) using Leu3a mAb (data not shown). Two different cultures of purified astrocytes were tested. As a control, human PBMC stained with the same mAb (23% positive cells). In mixed CNS cell cultures, about 13% of glial cells present in the monolayer were positively labeled with anti-CD4 mAb (data not shown).

Penetration of HIV-1 R5 and X4 Isolates into Human Astrocytes

Infection of astrocytes

We then examined the ability of HIV-1 R5 and X4 strains to penetrate astrocytes. Only cultures exhibiting a positive RT-PCR signal for CCR5 and CXCR4 and responding to MIP-1 β and SDF-1 α were selected. Viral DNA was measured by semiquantitative PCR assay using primers for the R/U5 region of the LTR designed to detect early steps in reverse transcription. DNA levels of HIV-1₉₅₃₃ (an M-tropic isolate) in astrocytes at 12 h pi (0.002 ± 0.001 HIV DNA units; $n = 2$) and at 18 h pi (0.003 ± 0.001 HIV DNA units; $n = 2$) were equivalent to those measured in astrocytes incubated with heat-inactivated virus (12 h: 0.002 ± 0.001 HIV DNA units; $n = 2$), as shown in Figure 5. These data indicate that HIV-1₉₅₃₃ failed to penetrate purified astrocytes. Similar results were obtained with astrocytes prestimulated with either IFN γ +TNF α , IFN γ +IL-1 β or TNF α +IL-1 β for 3 days (data not shown). As a positive control of viral entry, PCR products of the R/U5 region were detected in MDM after exposure to HIV-1₉₅₃₃ (12 h pi: 0.79 ± 0.01 vs 0.057 ± 0.001 HIV DNA units; infectious vs heat-inactivated virus; $n = 2$; Fig. 5). HIV infection was also monitored on astrocytes with the T-tropic HIV-1_{LAI} isolate known to use CXCR4. Neosynthesized viral DNA was not detected in cultured astrocytes after incubation with HIV-1_{LAI} (Fig. 5).

Susceptibility of primary mixed brain cells to HIV-1 R5 infection

In sharp contrast to the absence of viral penetration in isolated astrocytes, primary mixed cultures containing astrocytes, microglia, and neurons (as described in M&M) allowed entry of a M-tropic HIV-1 strain. As shown in Figure 5, the levels of the R/U5-PCR products in cells from primary mixed cultures incubated with inactivated virus were significantly lower than in cells exposed to infectious HIV-1₉₅₃₃, indicating that the virus penetrated and initiated reverse transcription steps in these cells. In all cases, pretreatment of cells with anti-CD4 mAb decreased HIV DNA levels (Fig. 5), indicating that virus entry was dependent on CD4. A similar inhibitory effect of pretreatment with anti-CCR5 mAb was observed (Fig. 5), but only in 2/4 primary mixed cultures. We also tested the susceptibility

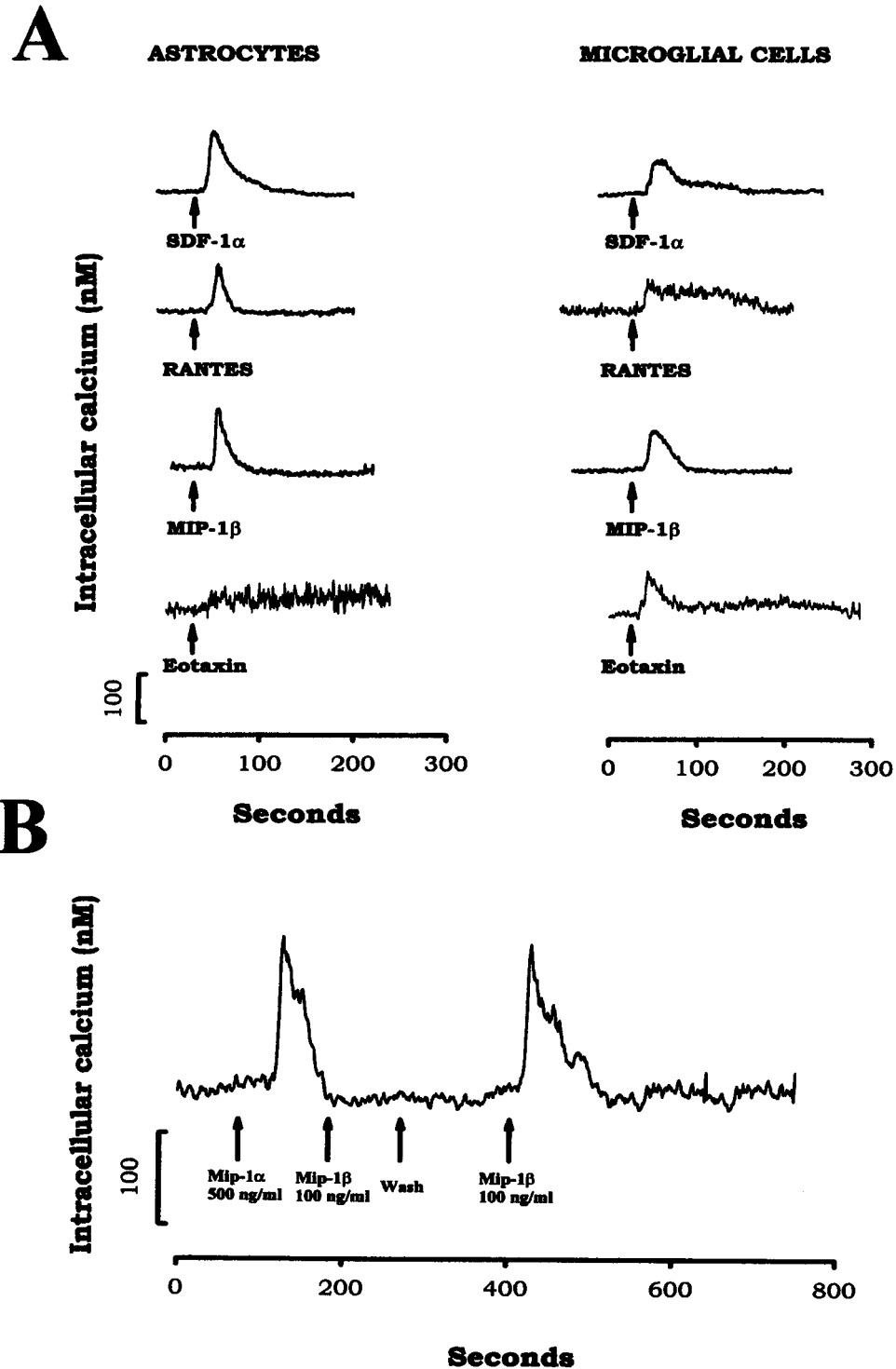


Fig. 3. Calcium flux in astrocytes and microglia stimulated with various chemokines. Intracellular Ca^{2+} was recorded as the relative fluorescence in Indo-1-loaded cells for 90 s. Arrows mark the time points at which chemokines were added at 100 ng/ml. Changes in free $[\text{Ca}^{2+}]_i$ were expressed by the ratio method using single-wavelength excitation (355 nm) and two-wavelength emission (405 and 490 nm). **A:** Traces recorded in astrocytes (or microglia) from one experiment

representative of 24 (5) for SDF-1 α , 19 (9) for MIP-1 β , and 16 (3) for RANTES and 25 (2) for eotaxin are shown. **B:** Cross-desensitization of the responses induced by 100 ng/ml of MIP-1 β with 500 ng/ml of MIP-1 α . Indo-1-loaded astrocytes were alternatively stimulated with the indicated chemokines, washed and subsequently tested for MIP-1 β -induced response.

of mixed CNS cells to HIV-1_{LAI} (X4 isolate) infection and found no difference in HIV-1_{LAI} DNA levels in cultures exposed to inactivated or infectious virus, in-

dicating an absence of new products of HIV-1 reverse transcription (Fig. 5). PBMC were used as positive control and HIV DNA was detected after infection with

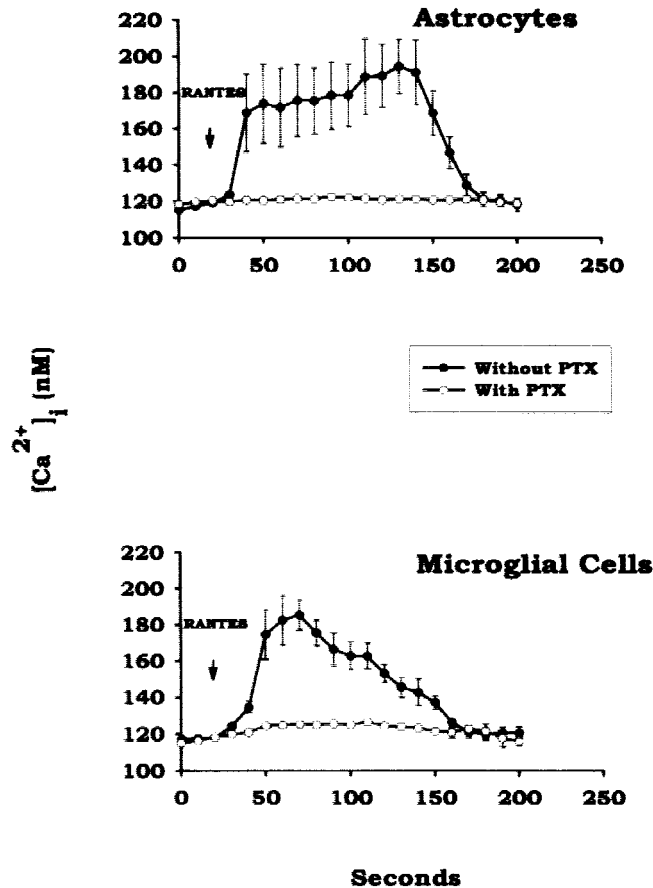


Fig. 4. Astrocytes and microglial cells respond to RANTES by the pertussis toxin-sensitive GPCR pathway. Indo-1-loaded cells were pretreated or not with PTX (250 ng/ml) and exposed to 100 ng/ml of RANTES. $[Ca^{2+}]_i$ were plotted versus time. The data points represent the mean \pm SD ($n = 5$).

HIV-1_{LAI}, but not in the presence of inactivated virus (12 h pi: 9.1 ± 1 vs 0.1 ± 0.01 HIV DNA units; infectious vs heat-inactivated virus; $n = 2$; Fig. 5).

DISCUSSION

We investigated whether chemokine receptors are required for viral entry into human CD4-negative astrocytes. We found that embryonic astrocytes expressed CXCR4 and CCR5 mRNA and proteins. Furthermore, their specific ligands stimulated calcium mobilization. CCR3 transcript was also detected but astrocytes failed to mobilize calcium after incubation with eotaxin. The distribution profile of the CXCR4, CCR5, and CCR3 proteins on embryonic astrocytes was similar to that observed on fetal astrocytes by Klein et al. (1999) but differed from the results obtained by Sabri et al. (1999), suggesting variations in the constitutive expression of chemokine receptors in cultured astrocytes. In fact, we found that only 25–36% of the astrocytes present in our cultures displayed increased intracellular calcium concentrations following chemo-

kine stimulation. This heterogeneity of responding cells could be detected within and between astrocyte cultures and might reflect variations in receptor expression between cells and, accordingly, the existence of subpopulations of astrocytes for these receptors. It may also be due to desensitization of receptors following repeated stimulation (Lefkowitz, 1993; Sabroe et al., 1997). Variations depending on developmental stage are also a possibility, but the role of chemokines in neuronal development (Araujo and Cotman, 1993; Halks-Miller et al., 1997; Horuk et al., 1997) suggests that the expression of their receptors must occur at early stage of ontogenesis. This is supported by the CXCR4- and SDF-1-knockout mice model showing altered migration of cerebellar neurons (Zou et al., 1998; Ma et al., 1998).

Primary astrocytes have been shown to express HIV transcripts after viral genomic transfection (Tornatore et al., 1994; Conant et al., 1994; Gorry et al., 1999; Ludwig et al., 1999). Similarly, astrocytoma cell lines can be infected by numerous HIV-1 isolates (Harouse et al., 1989; Neumann et al., 1995). In contrast, studies reporting HIV-1 entry into primary human astrocytes are scarce (Nath et al., 1995; Sabri et al., 1999). We observed that the M-tropic HIV-1₉₅₃₃ strain infected MDM via CCR5 but did not penetrate purified CCR5-positive astrocytes. In contrast, in primary mixed brain cell cultures, neosynthesized viral DNA was detected after exposure to HIV-1₉₅₃₃ and DNA levels were reduced with anti-CD4 and anti-CCR5 mAb, indicating that these two receptors are required for HIV-1₉₅₃₃ entry. Previous studies from our laboratory have indicated that HIV-1 antigen was present in both CD68-positive and GFAP-positive cells in mixed cultures infected with the same virus (Janabi et al., 1998). Using immunofluorescence staining, we observed that mixed cultures contained 13% of positive glial cells for CD4. In purified astrocytes, CD4 mRNA was detected by RT-PCR but CD4 protein was not present at the cell surface. In vivo CD4 mRNA was also found to be expressed in different areas of human brain (Erickson et al., 1991; Omri et al., 1994) and Funke et al. (1987) reported glial cells positive for CD4 protein in the cerebellum, thalamus, and pons. However, it has never been demonstrated that CD4-labeling was associated to GFAP expression. In contrast, in brain tissue, microglial cells were found to be positively stained for CD4 (Peudenier et al., 1991) and HIV-1 infection is dependent of CD4 in these cells (Jordan et al., 1991; He et al., 1997), as we observed in mixed culture. The comparison of purified astrocytes infection with mixed CNS cell infection suggests that CCR5-mediated penetration of HIV-1 into astrocytes would required CD4 antigen. As demonstrated by Wu et al. (1996), HIV-1 attachment to CCR5 was low in the absence of CD4 and the affinity of the interaction was increased in the presence of soluble CD4. Furthermore, Speck et al. (1999) demonstrated that CD4 provided in trans, induced

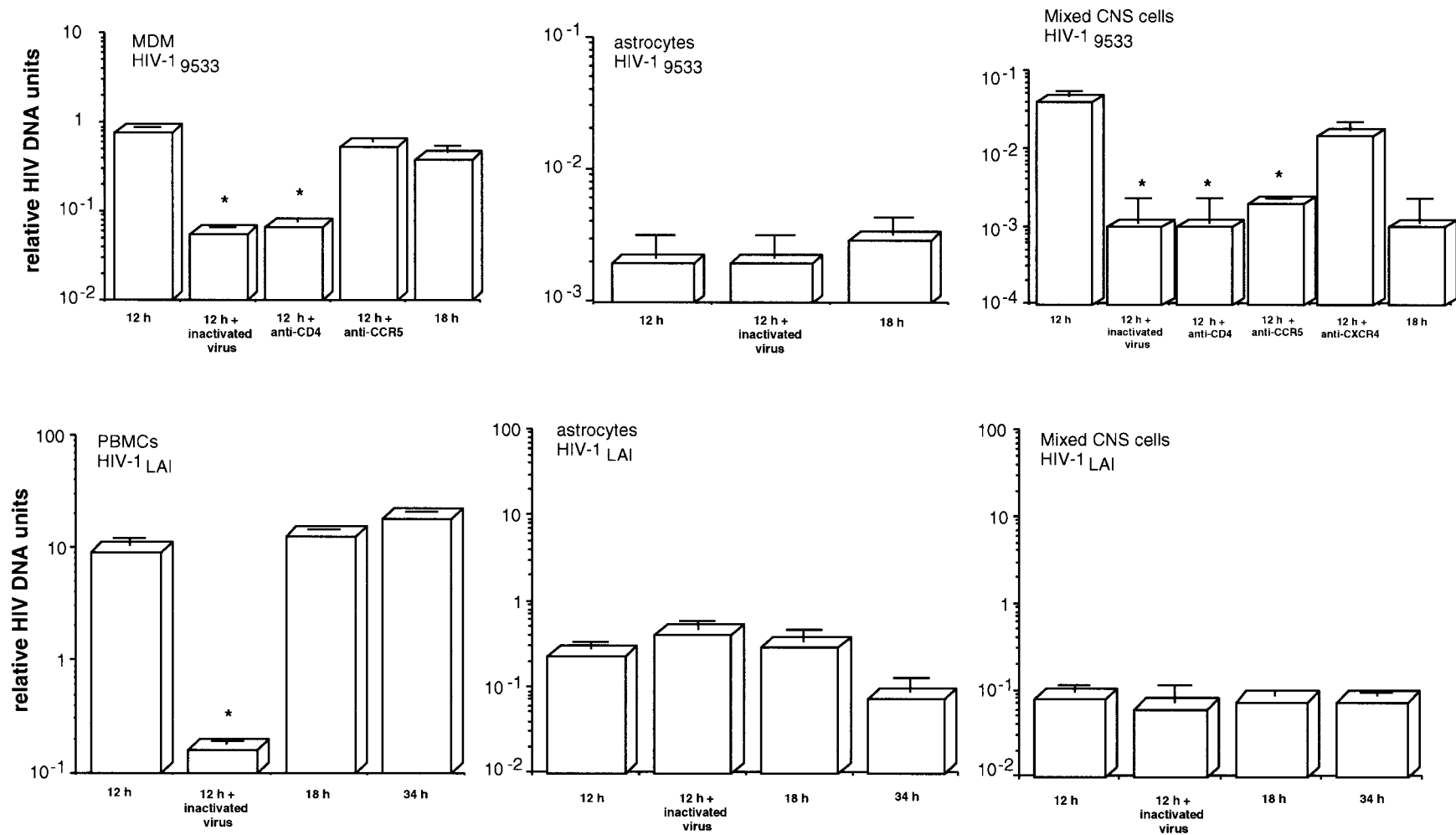


Fig. 5. Susceptibility of brain cells and leukocytes to infection by the HIV-19533 R5 and the HIV-1LAI X4 isolates. Purified cultures of astrocytes, mixed CNS cell cultures, or leukocytes were infected with HIV-1 overnight at 37°C and washed (12 h postinfection). Cells were lysed immediately, 6 h later (18 h pi) or 22 h later (34 h pi) to perform HIV LTR semiquantitative PCR assay. Baseline refers to cells pulsed with heat-inactivated HIV as control for DNA in the viral stock. For inhibition experiments, cells were incubated 45 min before infection with neutralizing antibodies. MDM were used as positive control of HIV-

19533 infection and anti-CD4 (Leu3a) and anti-CCR5 (2D7) inhibition. PBMC were used as positive control of HIV-1LAI infection. Results are presented as the mean \pm SEM of duplicate in one representative experiment out of three (for MDM, PBMC, and astrocytes) or two (for mixed CNS cells) performed with separate embryonic brain tissue specimens or blood donors. *Values are significantly different from HIV DNA units in infected MDM, PBMC, or mixed CNS cell 12 h pi according to a one-log difference.

HIV-1 infection of primary human CD4-negative astrocytes. In mixed CNS cell cultures, one can hypothesize that microglial cells supplied CD4 in trans to allow R5 HIV-1 penetration of astrocytes. The replication of HIV-1 M-tropic isolate on CD4-negative cells is however possible but required adaptation processes. Kolchinsky et al. (1999) demonstrated the adaptation of M-tropic isolate replication on CD4-negative canine cells expressing human CCR5 with concomitant changes in the V2 loop conformation of gp120.

The ability of HIV to use CXCR4 in the absence of CD4 in human fetal astrocytes has been described for a T-cell line adapted (TCLA) HIV-2 but not for primary X4 HIV-2 (Reeves et al., 1999). In our study, the TCLA HIV-1_{LAI} did not infect CXCR4-expressing astrocytes, suggesting that CD4⁺ astrocytes probably display variable susceptibility to HIV X4 strain infection. This might be related to viral strains bearing a fusion-competent Env conformation which would normally be expected to follow CD4 binding (Chan and Kim, 1998).

Since HIV-1₉₅₃₃ infection was inhibited by anti-CCR5 mAb in only 1/2 mixed brain cell cultures tested, this R5 viral isolate might use alternative coreceptors. APJ, GPR1 and CCR8 have been shown to support HIV-1 entry and are expressed in human CNS or brain-derived cells (Edinger et al., 1998; Jinno et al., 1998; Shimizu et al., 1999). Our data suggest that CCR8 was not present on astrocytes or microglia because I-309, its more potent agonist, did not induce Ca²⁺ responses in these cells, but the involvement of APJ and GPR1 remains possible. The ability of an M-tropic HIV-1 isolate to use additional coreceptors expressed on glial cells may lead to the biological selection for particular HIV-1 variants within the brain (Power et al., 1994; Chan et al., 1999) and the emergence of neurotropism or neurovirulence (Gabuzda et al., 1998).

In conclusion, whereas CCR5 mediated viral entry into mixed CNS cell cultures in conjunction with CD4, our results suggest that CCR5 cannot function as primary receptor for M-tropic HIV-1 strains in astrocytes. In vivo, HIV-1 might use CD4 supplied in trans by microglial cells to penetrate astrocytes. However, it cannot be ruled out that infection of astrocytes might occur through alternative mechanisms such as endocytosis (Hao and Lyman, 1999) or cell-to-cell transfer, as suggested by Kohleisen et al. (1992).

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