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Regulation of HIV-1 infection in astrocytes: expression of Nef, TNF-α and IL-6 is enhanced in coculture of astrocytes with macrophages

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'Restricted' human immunodeficiency virus type (HIV-1) infection of astrocytes is recognized *in vivo* in some pediatric and adult AIDS brains and *in vitro* in a small proportion of transfected primary fetal astrocytes. We investigated the extent of HIV-1_{JR-FL} expression in fetal astrocytes and macrophages cultivated alone or together. Peak HIV-1 p24 antigen titres in supernatant fluids of macrophage cultures were increased with monocyte/macrophages from certain donors and were higher when macrophages were cocultivated with astrocytes. Structural HIV-1 gene (*gp 41* and *pol*) products (protein and mRNA) were observed only in macrophages. Ten days after HIV-1_{JR-FL} infection, astrocytes in a monoculture were stained negative or only weakly positive (1–2+) for Nef, whereas in a coculture up to 100% of astrocytes displayed Nef staining (up to 4+) in the cytoplasm. The streptavidine-biotine-peroxidase technique with certain monoclonal antibodies to Nef (Ovod *et al*, 1992) was specific for infected astrocytes. The intensity of Nef staining was higher in astrocytes cultivated with monocyte/macrophages from certain donors. In the coculture, tumor necrosis factor-α (TNF-α) was expressed in the astrocyte cytoplasm earlier after coinfection with HIV-1 and cytomegalovirus (CMV) compared to infection with HIV-1 alone. Interleukin-6 (IL-6) was secreted spontaneously and transiently in uninfected cocultures, but in a prolonged fashion following HIV-1 and HIV-1/CMV infections. The interactions between HIV-1- and CMV-infected macrophages and astrocytes lead to upregulation of TNF-α and IL-6 and enhancement of productive HIV-1 infection of macrophages and of 'restricted' HIV-1 infection of astrocytes with implications for the pathogenesis of AIDS dementia.

Keywords: human immunodeficiency virus type 1; Nef; tumor necrosis factor-alpha; interleukin-6; cytomegalovirus; astrocyte

Introduction

In vitro HIV-1 infection of immature glial cells, primary fetal astrocytes (Tornatore *et al*, 1991) and glioma cell lines (reviewed by Volsky *et al*, 1992) has been achieved in a number of laboratories. In primary fetal astrocytes, after the initial productive phase HIV-1 infection assumes a persistent but reactivable form with viral RNA transcription 'restricted' to multiply spliced mRNA's, in particular *nef* transcripts, and with weak expression in rare (1/200–1/400) cells of Nef protein (Tornatore *et al*, 1994a) [except in up to 100% of cloned glial cells (Bracke-Werner *et al*, 1992)]. Viral mRNA expression in persistently HIV-1-infected astrocytes is similar to that in nonproductively infected mononuclear blood cells which is limited to the multiply spliced 2 kb mRNAs (Pomerantz *et al*, 1990). Nef is one of six HIV-1 accessory proteins and, despite an earlier impression of being a negative regulatory factor, it has a positive effect on virus replication in transformed and primary

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mononuclear cells (Subbramanian and Cohen, 1994). Different antigenic forms of Nef are distinguished by their subcellular localization in astrocytoma cells (Kohleisen *et al*, 1992). In lymphoid cells, Nef is initially found in the cytoplasm, but later translocates to the nucleus (Ranki *et al*, 1994). The 'restricted' nature of the astrocyte infection could be related to a cell-determined block in Rev function (Neumann *et al*, 1995) or due to global defects in the viral replicative cycle (Conant *et al*, 1994).

Monocyte/macrophages migrating into the central nervous system (CNS) in HIV-1-positive patients are central to AIDS neuropathogenesis because of the production of neurotoxins (Genis *et al*, 1992; Gendelmen *et al*, 1994; Nottet *et al*, 1995) and viral peptides, in particular gp120, which is toxic to glial cells (Benos *et al*, 1994), and their presumed ability to infect fetal astrocytes, which is surmised from the location of infected astrocytes in the brain areas infiltrated with monocyte/macrophages (Tornatore *et al*, 1994b). *In vitro* HIV-1 infection of fetal astrocytes has been accomplished by techniques, such as transfection and coculture with A3.01 T cells (Tornatore *et al*, 1991), which are not physiological in the CNS. We used macrophage/astrocyte cocultures in an attempt to develop a system suitable for investigation of mechanisms and regulation of astrocyte infection *in vivo*. We hypothesized that astrocyte infection would be upregulated either due to paracrine stimulation by tumor necrosis factor-α (TNF-α) and other cytokines, as shown previously with HIV-1 infection of monocyte/macrophages (Koyanagi *et al*, 1988; Poli and Fauci, 1992), or by cell-to-cell contact, as demonstrated with cocultures of macrophages and endothelial cells (Gilles *et al*, 1995). Cytomegalovirus (CMV) is a complicating factor in patients with AIDS (Rhodes, 1993) and, specifically, with AIDS dementia complex (ADC) (Fiala *et al*, 1993). CMV stimulates TNF-α, IL-1β (Haskill *et al*, 1993) and IL-6 production in macrophages (Pulliam *et al*, 1995). In the coculture system infected with HIV-$1_{JR\text{-}FL}$, TNF-α and IL-6 were upregulated, and Nef expression in astrocytes and HIV-1 production in marcophages were enhanced.

Results

Expression of Nef in astrocytes in HIV-1 and CMV-infected macrophage/astrocyte cocultures

Initial immunocytochemical experiments in (co-)cultures of astrocytes and macrophages established that structural HIV-1 antigens (p24, gp41 and gp120) could be detected only in macrophages but not in astrocytes. HIV-1 p24 antigen was detected in the supernatant of infected cultures beginning at 5 days P.I., rising to peak titre on days 12–15 P.I. The peak titre achieved with pure macrophage cultures (2×10^5 macrophages) was 1 ng/ml, whereas it was 20 ng/ml with astrocyte/macrophage cocultures (2×10^5 macrophages from an identical donor and 5×10^3 astrocytes). Macrophages from different donors produced variable peak p24 antigen titres (from 0.040 to 1 ng/ml).

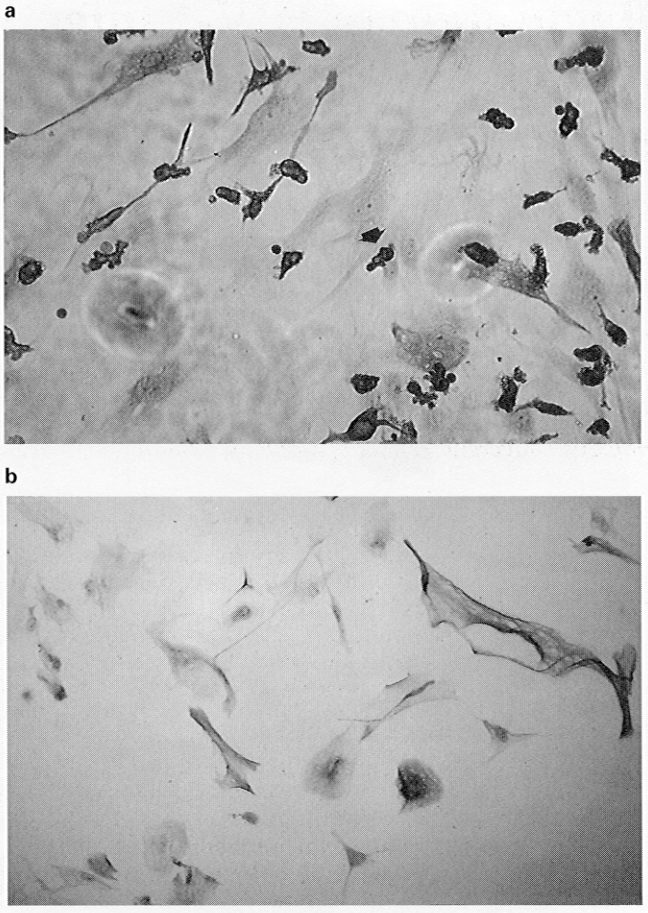

Figure 1 (a) HIV-1-infected coculture of primary human macrophages with human fetal astrocytes stained immunocytochemically with CD68+ monoclonal antibody and haematoxylin counterstain. Note 4+ staining of macrophages (small dark cells, e.g. at arrow-head) ($\times 200$). (b) Uninfected culture of human fetal astrocytes stained immunocytochemically with monoclonal antibody to GFAP ($\times 200$). Note variable (1 to 4+) staining of astrocytes.

Since previous *in vitro* studies showed expression of Nef in astrocytes (Brack-Werner *et al*, 1992; Kohleisen *et al*, 1992; Tornatore *et al*, 1994a), we examined infected cultures for the presence of Nef. HIV-1 infected monocultures of astrocytes were usually negative for Nef although with some astrocytes weak staining was noted. HIV-1-infected cocultures were positive for Nef by both immunocytochemical techniques employed. Nef staining was 4+ (on a scale 0 to 4+) in macrophages and 0 to 3+ in astrocytes with perikaryal or total cytoplasmic distribution. In some cocultures infected with HIV-1, 10 days P.I., up to 100% of astrocytes were positive for Nef using the Vectastain reagents and

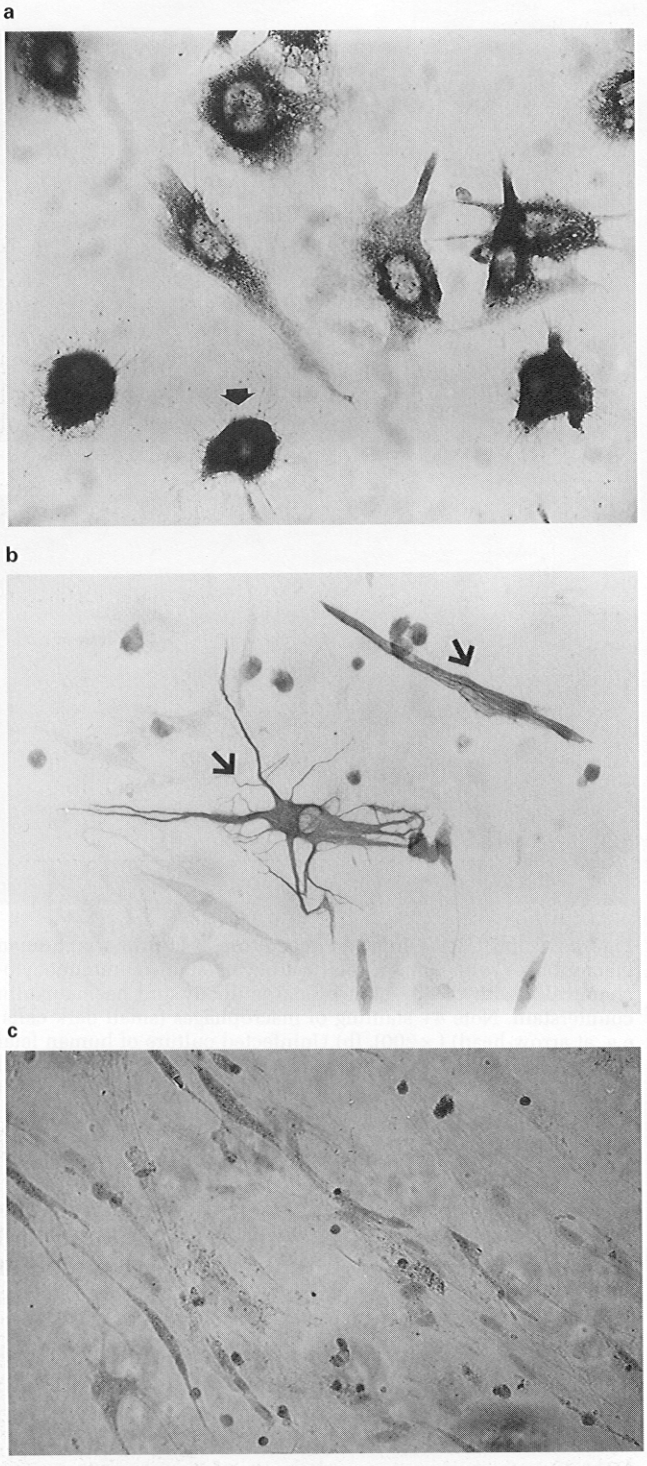

Figure 2 HIV-1-infected coculture of macrophages with astrocytes 10 days P.I. stained immunocytochemically with (a) Nef monoclonal antibody (No. 1534) and Elite ABC kit (×500), (b) Nef monoclonal antibody 3E6.2 and DAKO LSAB+ peroxidase kit (×500). Note in (a) strong (3–4+) perinuclear staining of seven astrocytes and pancellular staining of three macrophages (e.g. arrow-head), and in (b) total cytoplasmic staining of two process-bearing astrocytes (arrow); (c) Uninfected coculture of macrophages with astrocytes stained with Nef monoclonal antibody 3E6.2 and DAKO LSAB+ peroxidase kit (×400). The macrophages are not stained and the astrocytes reveal only minute deposits.

the antibody No. 1534 (Figure 2a). With these reagents, however, in some experiments even the uninfected astrocytes were weakly but nonspecifically positive (1 to 2+). Using the DAKO reagents with antibodies 3E6.2 and 3A2.2 (Ovod *et al*, 1992), Nef staining of astrocytes was specific for infected astrocytes with 4+ staining of the astrocyte cytoplasm, including fibrillary processes, and of macrophages (Figure 2b) and no staining of uninfected astrocytes and macrophages (Figure 2c). The results were astrocyte- and macrophage-donor dependent, i.e. astrocytes from donor no. 5 displayed negative staining for Nef in a coculture with macrophages from a particular donor, whereas astrocytes from donor no. 4 were positive in a coculture with macrophages from the same donor; also astrocytes from one donor cocultivated with macrophages from different donors displayed variable amounts of Nef staining (1-3+) (Table 1). Infected cocultures did not react with control normal mouse IgG. Expression of Nef and cytopathic effect in astrocytes 8 days P.I. were accelerated in CMV/HIV-1 coinfected cocultures (Figure 3a) compared to HIV-1 infected cocultures (Figure 3b).

In situ *hybridization for HIV-1 RNA in infected cocultures*

HIV-1 infected astrocyte/macrophage cocultures examined by *in situ* hybridization for HIV-1 RNA using the *pol* probe, revealed an accumulation of silver grains in some macrophages, whereas no silver grains were localized over astrocytes (Figure 4).

Upregulation of TNF-α in HIV-1- and CMV-infected cultures

Astrocytes were infected by CMV productively as demonstrated by (a) *in situ* hybridization, which was positive for CMV DNA in the nuclei, and (b) by transmission electron microscopy, that revealed aggregates of icosahedral nucleocapsids in the nuclei, enveloped herpesvirions in the cytoplasm, dense bodies in the extracellular space and severe vacuolar cytopathic changes of infected astrocytes 6–8 days P.I. (data not shown). CMV-infected astrocytes displayed cytopathic effects progressing from cellular swelling to contraction 8 days P.I. (Figure 3a) and lysis by 10–12 days P.I. (Figure 5b).

At 8 days P.I., in HIV-1 infected cocultures only rare macrophages were TNF-α positive (2+), whereas astrocytes were negative, while in HIV/CMV coinfected cocultures, TNF-α immunoreactivity was displayed by all macrophage (3+) and some astrocytes (1–3+). The staining intensities were greater on the cell surface than in the cytoplasm. At 10 days P.I., in HIV-1 infected cocultures TNF-α was immunostained in macrophages (4+) and astrocytes (2–3+) (Figure 5a), whereas, in HIV/CMV coinfected cocultures astrocytes had undergone cell lysis as a result of CMV infection but macrophages

remained 4+ positive (Figure 5b) (Table 2). Nef expression was, however, not inhibited when TNF-α receptor IL-1 receptor inhibitors were included in the medium of infected cocultures (data not shown).

Upregulation of secreted IL-6 in (infected) cocultures of macrophages and astrocytes

Neither macrophages nor astrocytes alone produced significant amounts of IL-6 in the medium in uninfected and HIV-1 infected cultures. Cocultivated uninfected macrophages and astrocytes released high levels of IL-6 with a maximum at 48 h after cocultivation of the two cell types. In virus infected cocultures, IL-6 production was upregulated in a more prolonged fashion with a peak at 48 h P.I. in CMV/HIV coinfected cells, and at ⩾72 h P.I. in HIV-1 infected cells (Figure 6).

Discussion

HIV-1 can be induced by transfection or coinfection to establish a persistent state of infection in fetal astrocytes during which the most abundant transcript is a multiply spliced 2 kb message specific for Nef and Rev, and rare cells express Nef weakly (Tornatore *et al*, 1994a). This study revealed that in HIV-1-infected fetal astrocyte/macrophage cocultures, especially with the use of macrophages and fetal astrocytes from certain donors, Nef expression in astrocytes involves up to 100% of astrocytes and is present in significant amounts. Using monoclonal Nef antibodies provided by Krohn and Ovod (Ovod *et al*, 1992) and the streptavidin-biotin-peroxidase-complex technique, nonspecific staining of uninfected astrocytes noted with some Nef antibodies

Table 1 Nef immunocytochemical (ICC) staining in cocultures of human fetal astrocytes (astrocyte) and monocyte/macrophages (macrophage)

Astrocyte donor[#]	*Number of macrophage donors*[1]	*ICC technique*	*Nef antibodies*	*Nef staining Macrophage*	*Astrocyte*	*Specificity*[2]
1	3	Vectastain	NIAID[3]	4+	0–3+	no
2	5	Vectastain	NIAID[3]	4+	0–3+	no
3	5	DAKO	Ovod and Krohn[4]	4+	0–3+	variable[5]
4	2	DAKO	Ovod and Krohn[4]	4+	1–3+	yes
5	1	DAKO	Ovod and Krohn[4]	4+	0	N.A.[6]

[1] The number of macrophage donors used with the astrocytes from a particular donor.
[2] Specificity of Nef staining in HIV-1 exposed astrocytes compared to unexposed astrocytes.
[3] NIAID Nef antibody No. 1534 as described in Materials and methods.
[4] Nef antibodies 3E6.2 and 3 A2.2 as described in Materials and methods.
[5] Variable specificity was encountered in cocultures performed in 16-well chamber slides.
[6] N.A. (not appropriate)

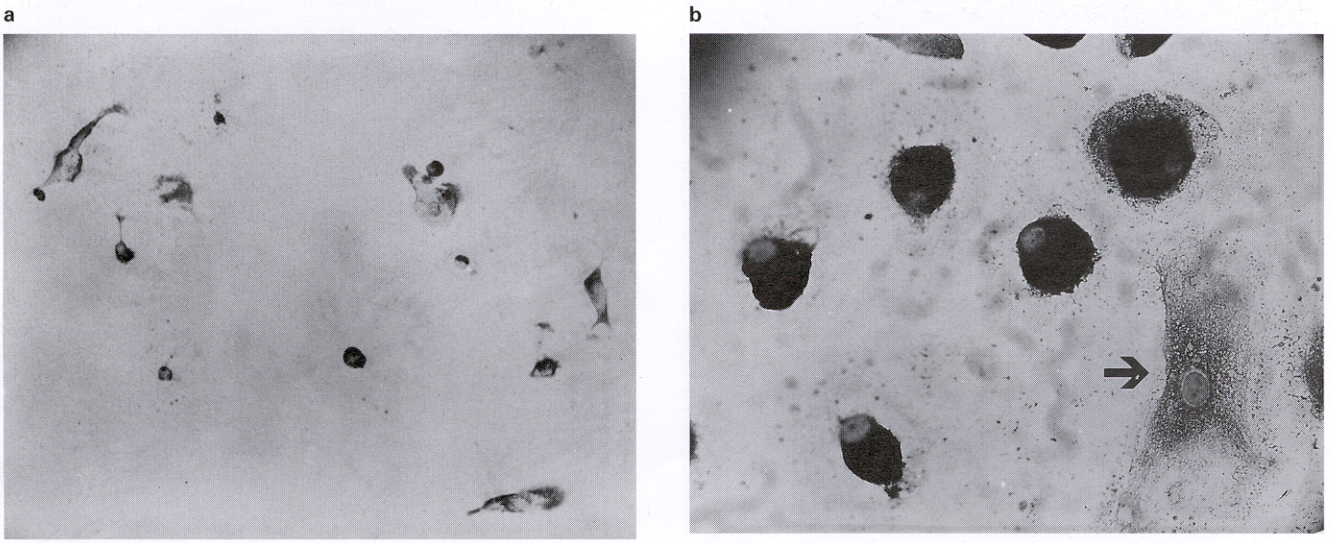

Figure 3 Coculture of macrophages with astrocytes 8 days P.I. stained by the Vetastain technique with Nef antibody (No. 1534) in (a) HIV-1/CMV-coinfected coculture (×500), and (b) HIV-1-infected coculture (×500). Note in (a) cytopathic effects (contraction of cell cytoplasm) and strong Nef staining in several astrocytes as well as strong staining of macrophages, and in (b) strong Nef staining of macrophages but weaker (1+ to 2+) Nef staining of an astrocyte without any cytopathic effect (arrow).

(Parmentier *et al*, 1992) is eliminated and the authenticity of Nef expression is confirmed.

Two proinflammatory cytokines, TNF-α and IL-6, are upregulated in infected cocultures in a prolonged fashion, especially after CMV/HIV coinfected. TNF-α is induced by LPS and IL-1β primarily in macrophages, but also in other cells (Vilcek and Lee, 1991). In human astrocytes *in vitro*, TNF-α expression is induced by LPS, IFN-γ and IL-1β in synergy (Chung and Benveniste, 1990). TNF-α induction by HIV-1 was considered controversial (Molina *et al*, 1990), but this is no longer disputed, especially in infected macrophage/astrocytes cocultures (Gendelman *et al*, 1994; Genis *et al*, 1992) and via gp120 in brain cells (Yeung *et al*, 1994. Stimulation of TNF-α by CMV is also documented (Haskill *et al*, 1993; Pulliam *et al*, 1995). TNF-α is considered to play an important role in neurological

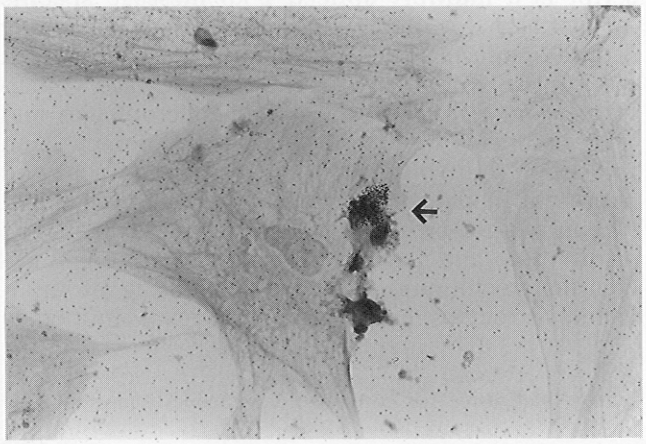

Figure 4 Combined immunocytochemical staining and *in situ* hybridixation for HIV-1 *pol* using immunoperoxidase stain with anti-GFAP (DAKO) antibody (×400). Note silver grains localized exclusively over one macrophage (arrow).

Table 2 Tumor necrosis factor-α (TNF-α) staining in HIV-1- and CMV- infected macrophage/astrocytes cocultures

Days P.I.	*Culture*	*Infection*	*TNF-α reaction in macrophages*	*TNF-α reaction in macrophages*
8	Coculture[1]	HIV	2+	0+
8	Coculture	HIV/CMV	3+	1–2+
10	Coculture	HIV	4+	2–3+
10	Coculture	HIV/CMV	4+	lysis

[1] Coculture of macrophages with astrocytes

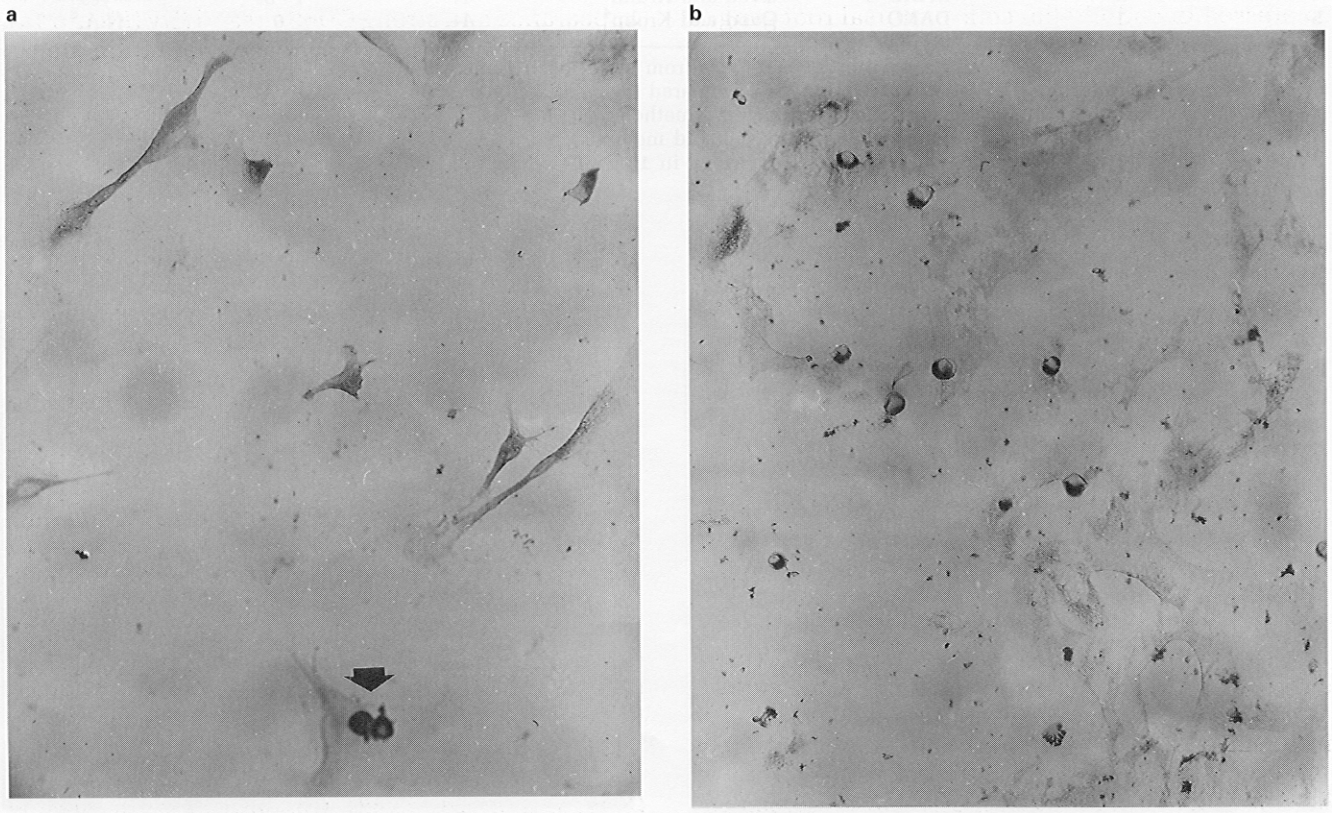

Figure 5 Immunocytochemical staining with rabbit TNF-α antibody of a coculture of macrophages with astrocytes (a) 10 days post-HIV infection (×240), and (b) 10 days post-CMV/HIV coinfection (×240). Note in (a) 4+ staining of two macrophages (arrow-head) and weak (1 to 2+) staining of astrocytes (without cytopathic effect), and in (b) 4+ staining of the many macrophages and lysis of most astrocytes with a residuum of astrocyte 'ghosts'.

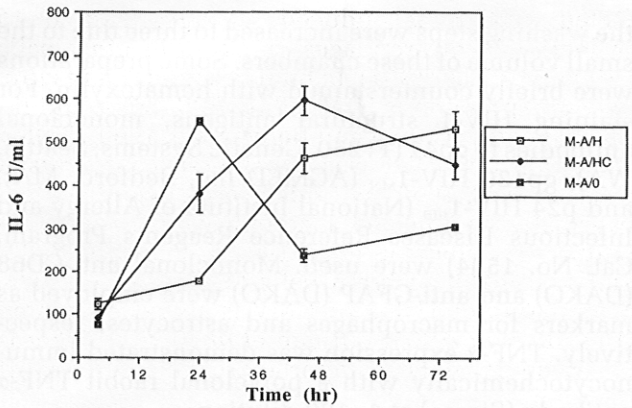

Figure 6 Production of IL-6 in cocultures of macrophages with astrocytes (MA) infected with HIV-1 (M-A/H), HIV-1 and CMV (M-A/HC), and sham-infected (M-A/0). The results are presented in IL-6 Units/ml as the mean ± S.D.

disease of AIDS patients (Vitkovic *et al*, 1994, Wesselingh *et al*, 1994) by enhancing HIV-1 replication (Poli and Fauci, 1992), reactivating productive infection (Tornatore *et al*, 1991), and inducing astrocytosis and oligodendrocyte death (Wilt *et al*, 1995). IL-6 stimulates HIV-1 expression in latently infected monocytes (Poli and Fauci, 1992; Vitkovic *et al*, 1991). In AIDS patients IL-6 is expressed in endothelial cells of dorsal root ganglia (Yoshioka *et al*, 1994). IL-6 has multifaceted effects in neurons (ranging from vacuolization (at 1 ng/ml) (Yeung *et al*, 1994) to enhanced survival (at a concentration of 100 U/ml) (Moroni and Rossi, 1995), and in astrocytes, which are stimulated to proliferate (Selmaj *et al*, 1991). The upregulation of TNF-α and IL-6 production in coinfected cocultures of macrophages and astrocytes *in vitro* may have implications for AIDS dementia with respect to enhancement of 'restricted' astrocyte infection and direct toxicities of these two cytokines.

Numerous perivascular and intraparenchymal monocyte/macrophages are characteristic findings in the AIDS brain (Rhodes, 1993), and they can be infected by HIV-1 (Koenig *et al*, 1986; Wiley *et al*, 1986), CMV, or both (Fiala *et al*, 1993). *In situ* hybridization using full-length genomic (Tornatore *et al*, 1994b) and *nef* RNA probes (Saito *et al*, 1994) in the brains of children with AIDS, and *in situ* hybridization and immunocytochemistry (with a majority of those monoclonal antibodies used in this study) in adult AIDS brains (Ranki *et al*, 1995), suggest that restricted, and sometime productive, infection of astrocytes located in the vicinity of infiltrating macrophages is a contributory mechanism in AIDS encephalopathy. The interactions between HIV-1 infected monocyte/macrophages and astrocytes can be analyzed in the coculture system with respect to cell-to-cell and cytokine-mediated mechanisms. Enhancement of Nef expression appears to be dependent on cell-to-cell contact as well as on extracellular factors, as TNF-α and IL-1 inhibitors did not abolish Nef expression. Taken together, the present data suggest that the interactions between HIV-1- and CMV-infected macrophages and astrocytes lead to upregulation of TNF-α and IL-6, and to enhancement of productive HIV-1 infection of macrophages and 'restricted' or even productive HIV-1 infection of astrocytes. We speculate that the infection of astrocytes is regulated at the stage of entry by cell-to-cell contact with macrophages and, at the stage of Nef expression, by soluble factors, including TNF-α, IL-6 and other cytokines.

Materials and methods

Cell culture and virus

Monocyte/macrophages Mononuclear cells isolated by the Ficoll-Hypaque technique from venous blood of healthy (HIV-1 and hepatitis seronegative) volunteers were allowed to attach for 4 – 24 h to poly-D-lysine coated 12 mm diameter coverslips (200 000 cells/coverslip) in 24 well culture plates (Falcon Plastics, Oxnard, CA), washed three times, and incubated in RPMI medium with 5% human serum and 15% fetal bovine serum for 7 – 10 days until they differentiated into macrophages (Baldwin *et al*, 1993) with a characteristic spread-out and round appearance and strong CD68+ reactivity (Figure 1a). In some experiments macrophages were cultivated in 8- or 16 well chamber slides (Lab-Tek chamber slide, Nunc Inc, Naperville, Il 60566) or 8 well SuperCell slides (Erie Scientific Co, Portsmouth, NH).

Human fetal astrocytes 'referred to as astro-cytes' Human neural cells obtained by mechanical disruption of fetal brain tissues were cultured in Dulbecco's modified Eagle's medium Ham's F-12 (1 : 1 mix) (DMEM/F-12) with 10% fetal bovine serum. Astrocytes were prepared by orbital shaking of these cultures as described by Tornatore *et al* (1991) and were cultured in 75 cm^2 cell culture flasks at passages 2 – 8, when they were >99% glial fibrillary acidic protein (GFAP) antibody positive (Figure 1b), except for the cells from donor 5 (Table 1). Five thousand astrocytes were cocultivated on each 12 mm diameter coverslip with macrophages. In the coculture, small round macrophages could be distinguished from the larger star-shaped astrocytes by their size and verified by staining with CD68 (KP-1) monoclonal antibody (DAKO Corporation, Carpinteria, CA) (Figure 1a). In some experiments, cocultures were performed in 8 well and 16 well slides.

HIV-1 Supernatant cell-free HIV-1_{JR-FL} virus stock (Koyanagi *et al*, 1988) was prepared in normal human umbilical cord mononuclear cells stimulated by IL-2 (10 U/dl) and phytohemagglutinin-A (0.5 μg/ml). The supernatant of infected cultures was harvested 10 days post-infection (P.I.) and its

titre measured by EIA p24 assay (Coulter Corporation, Hialeah, FL) to be 4.2 ng/0.1 ml before storage at −70°C.

Cytomegalovirus A CMV strain isolated from the blood of a patient with AIDS was propagated in human fibroblasts and used as freshly harvested supernatant medium titrating 10^4-10^5 PFU/ml.

HIV-1 and CMV infections of cocultures and use of cytokine receptors inhibitors HIV-1 infection was carried out with 0.1 ml of HIV-1_{JR-FL} stock 1 or 2 days after coculture of astrocytes with macrophages. CMV/HIV-1 coinfection was performed by adding equal volumes of each virus simultaneously. After 1 h of virus adsorption, the cells were washed four times and incubated in DMEM/F12 with 10% fetal bovine serum for 8 to 10 days followed by immunocytochemistry. In some experiments soluble human TNF-a receptor (p80) linked to the Fc portion of human IgG1, recombinant IL-1 receptor, antibody to human TNF-α receptor or antibody to IL-1 receptor (all from Immunex Corporation, Seattle, WA) were included in the medium of infected cocultures at concentrations of 2, 10 or 50 μg/ml during the experiment (10 days).

Immunocytochemistry for HIV-1 proteins, cell markers and TNF-α Mono- and cocultures were fixed with cold acetone:methanol (1:1) for 30 min and immunocytochemical staining was performed either using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) with an avidin-biotin-peroxidase complex or the DAKO LSAB+ Peroxidase Kit (Universal) (DAKO) with the streptavidin-biotin-peroxidase complex. Diaminobenzidine was used as the chromogen in both techniques in combination with H_2O_2 substrate. The antibody was omitted for control staining of cells, with normal mouse IgG substituted. Using the Vectastain technique, one of six mouse monoclonal anti-HIV-1_{JR-CSF} Nef antibodies (National Institute of Allergy and Infectious Diseases Reference Reagents Program, Cat. No. 1534 to 1539) was used as the primary antibody; with the DAKO technique, one of nine different monoclonal Nef antibodies, 3F2.2, 3D2.2, 2E3.2, 2 F2.2, 3E6.2, 3A2.2, 2E4.2, 3A2.2, 2H12.2 from K Krohn and V Ovod, University of Tampere, Finland (Ovod *et al*, 1992), was utilized. The primary antibodies were applied at a dilution 1:100 for 30 min at room temperature. The staining procedure was usually performed in 24 well culture plates with two 5 min washes between the steps of the procedure. In 8 well and 16 well chamber slides, the washing steps were increased to three due to the small volume of these chambers. Some preparations were briefly counterstained with hematoxylin. For staining HIV-1 structural antigens, monoclonal antibodies to gp41 (1:250, Genetic Systems, Seattle, WA), gp120 HIV-1_{MN} (AGMED Inc, Bedford, MA), and p24 HIV-1_{IIIB} (National Institute of Allergy and Infectious Diseases Reference Reagents Program, Cat. No. 1504) were used. Monoclonal anti-CD68 (DAKO) and anti-GFAP (DAKO) were employed as markers for macrophages and astrocytes, respectively. TNF-α expression was demonstrated immunocytochemically with a polyclonal rabbit TNF-α antibody (Sigma) at 1:100 dilution.

In situ *hybridization* (ISH) for detection of HIV-1 RNA was performed as described previously (Shapshak *et al*, 1990; Yoshioka *et al*, 1992) using sense and antisense riboprobes specific for the *EcoR*I internal fragment of the polymerase gene of HIV-1 labeled with [^{35}S]UTP. After 5–14 days of autoradiography, slides were developed and stained with hematoxylin and eosin. CMV ISH was performed with the use of a biotinylated DNA probe and hybridization reagents from the Pathogene Kit (Enzo Diagnostics, NY) (Fiala *et al*, 1993).

IL-6 assay

IL-6 was measured by an enzyme-linked immunosorbent assay technique described previously (Miles *et al*, 1990) in the supernatant of infected or uninfected cultures. Three replicate dishes (each with 5×10^5 macrophages, 4×10^4 astrocytes, or both) were used with each experimental treatment.

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