Media components influence viral gene expression assays in human fetal astrocyte cultures

Micheline McCarthy[1,5], Charles Wood[1,3,5], Larisa Fedoseyeva[1,5] and Scott R Whittemore[2,4]

Departments of [1]Neurology, [2]Neurological Surgery, and [3]Microbiology and Immunology, University of Miami School of Medicine; [4]The Miami Project to Cure Paralysis; [5]Veterans Administration Medical Center, Miami, FL 33101, USA

In vitro neurovirological studies of viral infectivity or viral gene expression may be confounded by the multiple neural cell types and/or fibroblast contamination present in early passage cultures prepared from dissociated human central nervous system (CNS) tissue. We have developed highly enriched astrocyte cultures for neurovirological study by culturing in a serum-free defined medium, B16, supplemented with basic fibroblast growth factor (FGF-2). Subculture in this medium selects against fibroblast proliferation and favors sustained proliferation of a highly enriched glial fibrillary acidic protein (GFAP)-positive cell population. These astrocytes support productive replication of cytomegalovirus (CMV) and transient expression of transfected CMV and human immunodeficiency virus type 1 (HIV-1) viral promoters. By comparison, CNS cultures developed in standard serum-containing medium initially contain predominantly astrocytes, but show increasing contamination with fibroblasts with sequential passage. These cultures support CMV viral synthesis in both fibroblasts and astrocytes, cell types distinguishable only by immunostaining for cell specific antigen. CMV or HIV-1 promoter activities, quantitated by transient gene expression assays, are distinctly lower in CNS cultures maintained in serum-containing medium.

Keywords: astrocytes; cytomegalovirus; HIV long terminal repeat; neuroglia; fibroblasts

Introduction

To investigate viral infectivity or gene expression in neural cells, many *in vitro* studies assay culture supernatants or cell monolayer lysates. These studies may be confounded by the multiple cell types present in primary or low passage cultures prepared from dissociated human central nervous system (CNS) tissue (Kennedy *et al*, 1980; Lee *et al*, 1992). *In vitro* studies of the neuropathogenesis of human immunodeficiency virus type 1 (HIV-1) infection in such cultures had emphasized the microglia as the site of infection (Watkins *et al*, 1990; Sharpless *et al*, 1992). The pathogenetic role of HIV-1 infection of astroglia is much more controversial (Blumberg *et al*, 1994). Studies in 'astroglial' cells have often used transformed glial cell cultures (Chiodi *et al*, 1987; Dewhurst *et al*, 1987; Brack-Werner *et al*,

1992; Kohleisen *et al*, 1992; Jault *et al*, 1994), with immortalized cells expressing antigenic markers for astrocytes. However, recent investigations utilized primary or early passage dissociated fetal CNS cell cultures enriched for astrocytes (Tornatore *et al*, 1991, 1994; Ho *et al*, 1991), and have observed 'restricted' HIV-1 infection in the astrocytes (Blumberg *et al*, 1994). Arguably, early passage astrocytes more closely mimic astrocytes *in vivo* than do immortalized cell lines, whose sustained growth reflects altered cellular transcriptional control and regulatory gene expression. Unfortunately, primary astrocytes are difficult to obtain in sufficient quantity and purity with which to conduct many neurovirological studies.

Our studies of herpesvirus-HIV-1 interactions in the developing CNS (McCarthy *et al*, 1994, 1995) have necessitated development of highly enriched human fetal astrocyte cultures that sustain assays for viral infectivity or viral gene expression. These assays are sensitive to the purity of the initial cell population and the potential outgrowth of fibro-
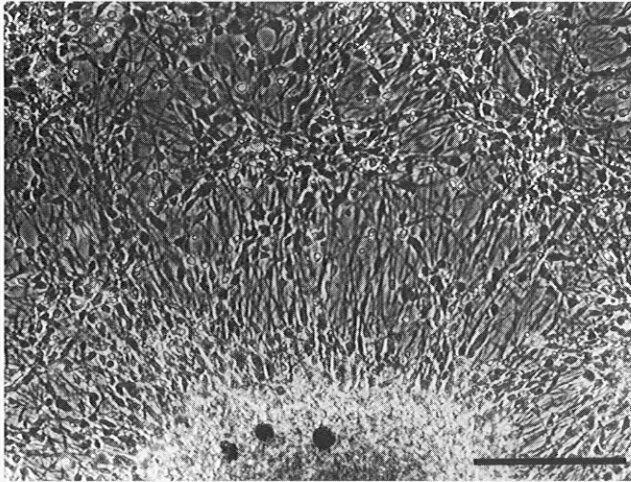
276

Figure 1 Phase contrast micrograph taken on day 9 after the initial culture of rostral CNS tissue, seeded in DF-FBS but maintained in B16-FGF-2. Cells form clusters with radiating processes along which single cells migrate. Magnification bar = 100 μm.

blasts. Previously, cultures of dissociated human fetal CNS have been enriched for astrocytes by high speed shaking to remove glial precursors or 'spongioblasts', (Major and Vacante, 1989), by repeated subculture using trypsin dissociation (Lee *et al*, 1992), or by cytosine arabinoside treatment (Kennedy and Fok-Seang, 1986) to eliminate fibroblasts. We now have developed highly enriched astrocyte cultures for neurovirological study by adapting a serum-free defined medium, B16, originally described for culture of rat hippocampal neurons (Brewer and Cotman, 1989). With serial passage, these cultures provide sufficient numbers of astrocytes from less than 1 g first trimester fetal rostral CNS tissue. As demonstrated in the present study, the culture conditions of astroglial populations may markedly affect the outcome and confuse the interpretation of neurovirological assays that sample cell culture supernatants or lysates.

Results

Characterization of fetal astrocyte cultures

Dissociated fetal CNS cells initially formed a heterogeneous culture, with aggregates of cells forming scattered cell clusters (Figure 1). These clusters radiated long processes which were glial fibrillary acidic protein (GFAP)- or neuron specific enolase (NSE)-positive; cells migrated along these processes. Cultures fed with B16-FGF-2 (B16 supplemented with basic fibroblast growth factor) had cells with more distinct and fibrous processes, but otherwise developed similarly to those cultured in DF-FBS (Dulbecco's Modified Eagle's Medium plus Ham's F12 medium (GIBCO BRL, Gaithersburg, MD) in equal proportions supplemented with 10% (v/v) fetal bovine serum). On initial subculture of cells

grown in either medium, GFAP expression ranged from 75%–95%.

Upon serial passage, distinct differences in cell morphology and the expression of antigens specific for astrocytes and fibroblasts were observed in cultures maintained in DF-FBS (Figure 2) or B16-FGF-2 (Figure 3). Process-bearing as well as flat, polygonal GFAP-positive cells initially dominated all cultures (Figures 2a, 3a), but GFAP expression gradually disappeared from cultures serially passaged in DF-FBS (Figures 2d,g). Cultures in DF-FBS had broad, flat polygonal cells, plus smaller polygonal cells with fine processes. Both perinuclear and extracellular fibronectin expression typical of fibroblasts was apparent and extensive in DF-FBS cultures, as early as the first subculture (Figure 2b), and fibronectin positive cells dominated the cultures by passage 5 (Figures 2g,h). In contrast, cultures in B16-FGF-2 initially had predominantly smaller polygonal cells with processes, but flat cells with more extensive cytoplasm were present and increased in frequency with serial passage (Figure 3). In cultures maintained in B16-FGF-2, scattered fibronectin positive cells were seen on initial subculture (Figure 3b), and occurred infrequently with serial passage (Figures 3e,h). With serial passage in B16-FGF-2, cultures sustained near confluent GFAP expression (Figures 3d,g). These patterns of GFAP and fibronectin expression also occurred in cultures passaged at higher dilution (2×10^5 cells cm^{-2} *versus* 5×10^5 cells cm^{-2}) and thus subcultured less frequently, approximately biweekly *versus* weekly.

To confirm this apparent overgrowth of fibroblasts in the serially passaged DF-FBS cultures, all cultures were also double immunostained for prolyl-4-hydroxylase, an enzyme found in cells that synthesize collagen (Janin *et al*, 1990), as well as for GFAP (Figure 4). Intense perinuclear prolyl hydroxylase staining was observed in the DF-FBS cultures (Figure 4e), as was extensive fibronectin staining (Figure 4b). Two color immunofluorescence confirmed that fibronectin-positive cells corresponded to prolyl-4-hydroxylase-positive cells (data not shown). With serial passage, broad, flat prolyl hydroxylase-positive cells proliferated in the DF-FBS cultures (Figure 4e). In contrast, by P2 only scattered, infrequent prolyl hydroxylase-positive cells appeared in the B16-FGF-2 cultures (data not shown). Two color immunofluorescence confirmed that GFAP-positive cells were distinct from cells expressing fibronectin (compare Figures 4a,b) or prolyl hydroxylase (compare Figures 4d,e). However, many GFAP-positive cells were morphologically indistinguishable from fibronectin- or prolyl hydroxylase-positive cells (compare Figures 4c with 4a,b and 4f with 4d,e respectively), underscoring the need for cell-specific antigens to accurately determine cell phenotype.

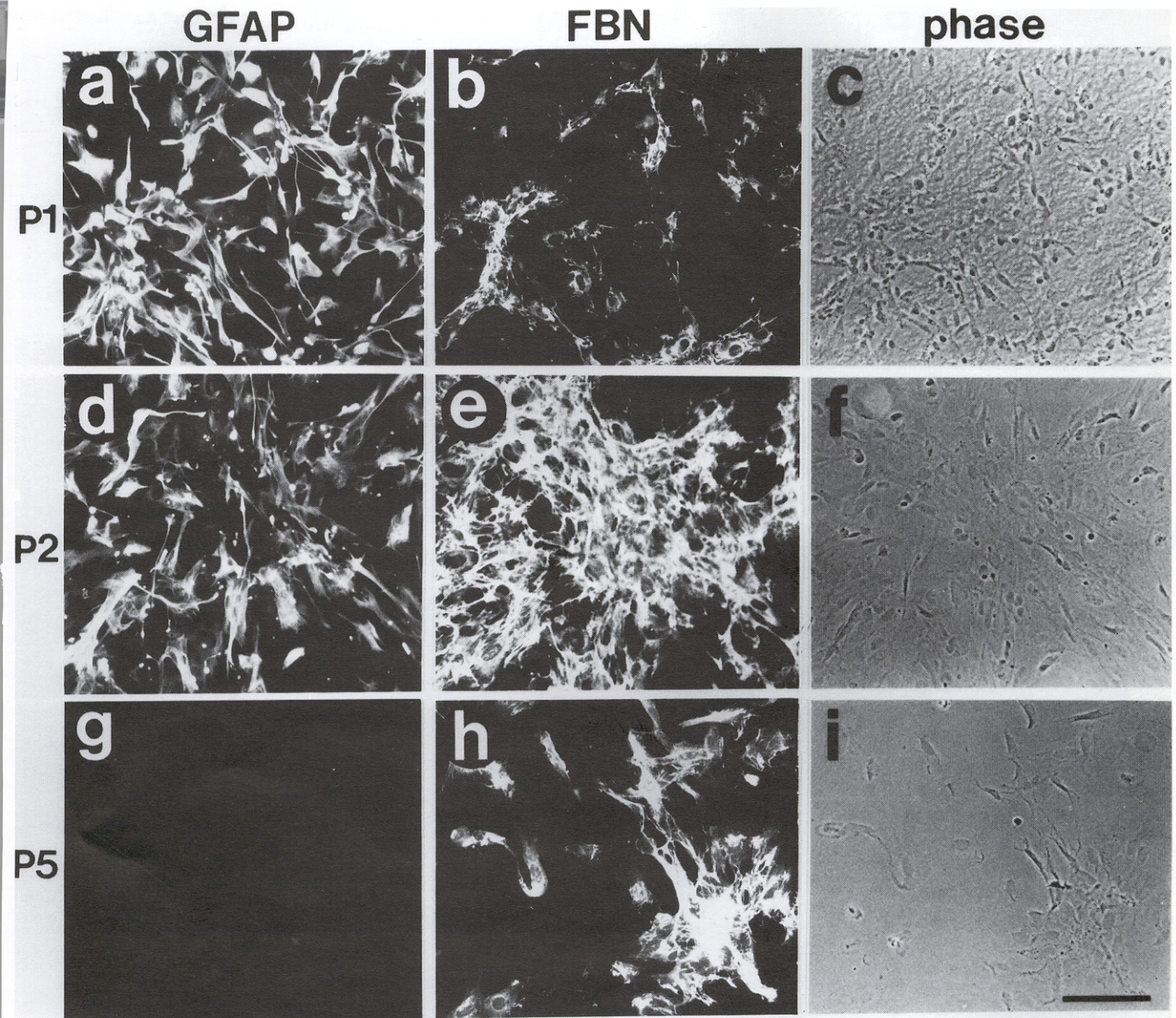Cultures maintained in B16-FGF-2 or DF-FBS did not differ dramatically in the expression of antigens

GFAP	FBN	phase

Figure 2 Immunofluorescent staining of rostral human fetal CNS cultures maintained with serial passage in DF-FBS. Cells were analyzed by phase contrast microscopy (c,f,i) and two color immunofluorescence at the indicated passage number (P1, P2, or P5) for glial fibrillary acidic protein (GFAP) (a,d,g) or fibronectin (FBN) (b,e,h). Identical results were observed in six independent cultures analyzed similarly. Magnification bar = 100 μm.

specific for other cell types. After initial subculture, none of the cultures expressed NSE. At early passages, rare cells had the highly branched morphology suggestive of oligodendrocytes and expressed 04-immunoreactivity (data not shown). Refractile, granular cells expressing CD68, a marker for microglia (Lee *et al*, 1992), constituted about 1% of cells, and were usually detected in cultures from fetal specimens of at least 70d gestation (data not shown). These microglia disappeared with serial passage in both DF-FBS and B16-FGF-2 media. Small, round, refractile, cells with bipolar processes were prominent in cultures from approximately 15% of fetal CNS specimens. These cells tended to

layer over the larger and flatter fibroblasts or astrocytes. These bipolar cells had the morphological characteristics of 'spongioblasts', pluripotent glial precursor cells derived from primitive ventricular epithelium (Shein, 1965). The cells expressed the A2B5 surface ganglioside antigen (data not shown), and varying proportions of these cells also expressed GFAP. Previous studies had shown that these bipolar cells could be removed from the cultures by high speed shaking (Major and Vacante, 1989), or the bipolar cells would usually disappear with serial two to four passages (Lee *et al*, 1992). Consistent with these reports, in our fetal CNS cell cultures, these cells would usually disappear by the
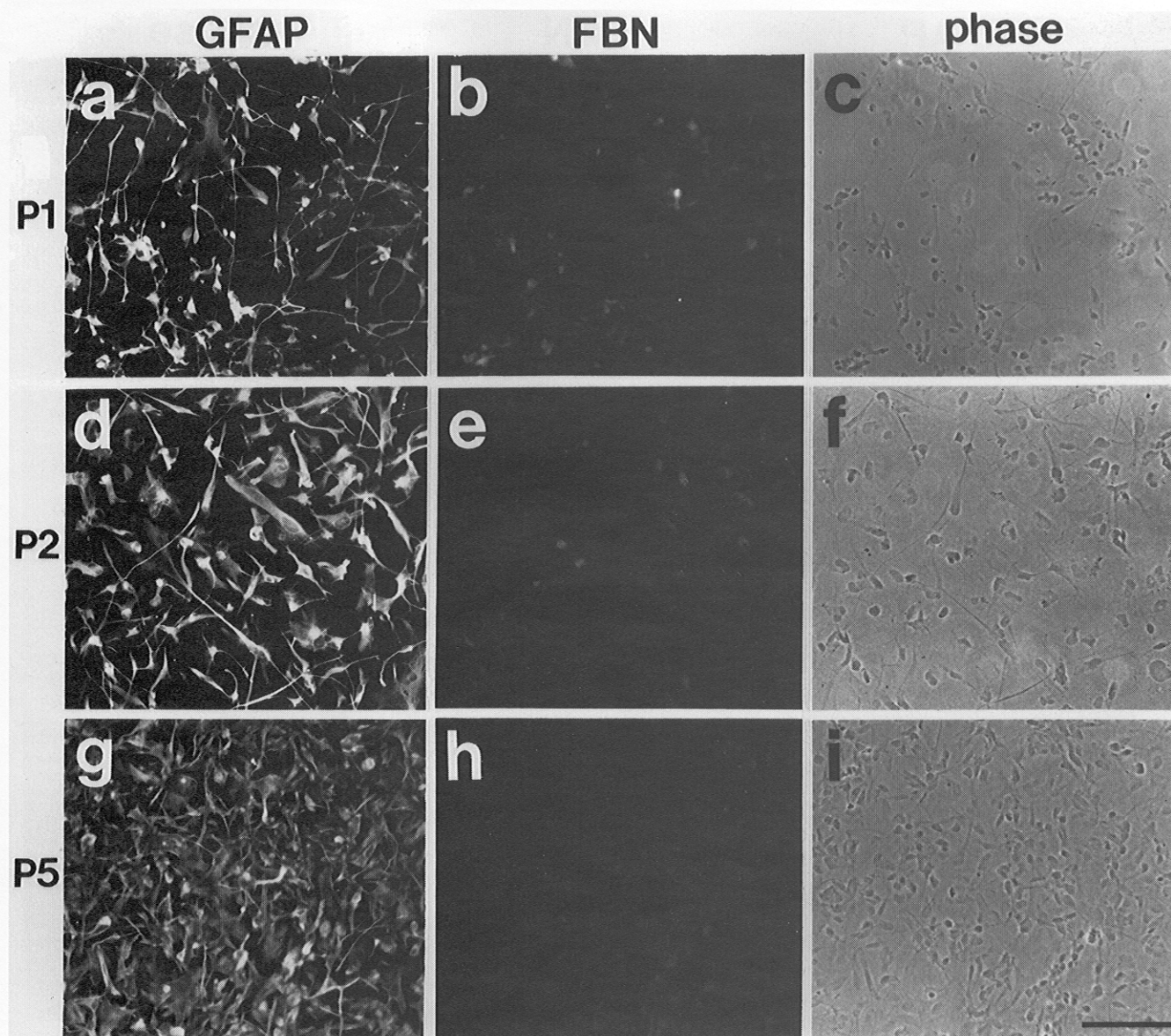
Figure 3 Immunofluorescent staining of rostral human fetal CNS cultures maintained with serial passage in B16-FGF-2. Cells were analyzed by phase contrast microscopy (c,f,i) and two color immunofluorescence at the indicated passage number (P1, P2, or P5) for glial fibrillary acidic protein (GFAP) (a,d,g) or fibronectin (FBN) (b,e,h). Identical results were observed in 16 independent cultures analyzed similarly. Magnification bar = 100 μm.

third passage. In cultures established from approximately 5% of our fetal CNS specimens, these bipolar cells persisted in culture for more than four serial passages. The appearance or survival of the bipolar cells was not affected by the culture medium. Such cultures were not used for viral infectivity or gene expression assays.

CMV infection of fetal astrocyte cultures
CMV productively infected fetal astrocyte cultures that had been maintained in B16-FGF-2 or DF-FBS at P2-P8. In cultures infected at low moi (0.1 pfu cell⁻¹), cytopathic effects (cpe) were visible within 36 h. Cells appeared swollen, slightly refractile, and

had enlarged nuclei with prominent nucleoli. CMV-specific nuclear antigens could be detected in either GFAP-positive (Figures 5a,b) or fibronectin-positive (Figures 5c,d) cells. The GFAP staining pattern suggested cytoplasmic contraction of the intermediate filaments in the swollen cells. Nuclear antigens appeared in scattered foci within 24 h post infection, and spread diffusely throughout the monolayer. The cpe corresponded to CMV antigen expression in cells. All cultures produced infectious CMV in either supernatants or cell lysates, with titers of 10^5-10^6 pfu ml⁻¹ (approximately 0.1–1 pfu cell⁻¹) determined at day 5 post infection. Despite diffuse CMV involvement and cpe, monolayers remained
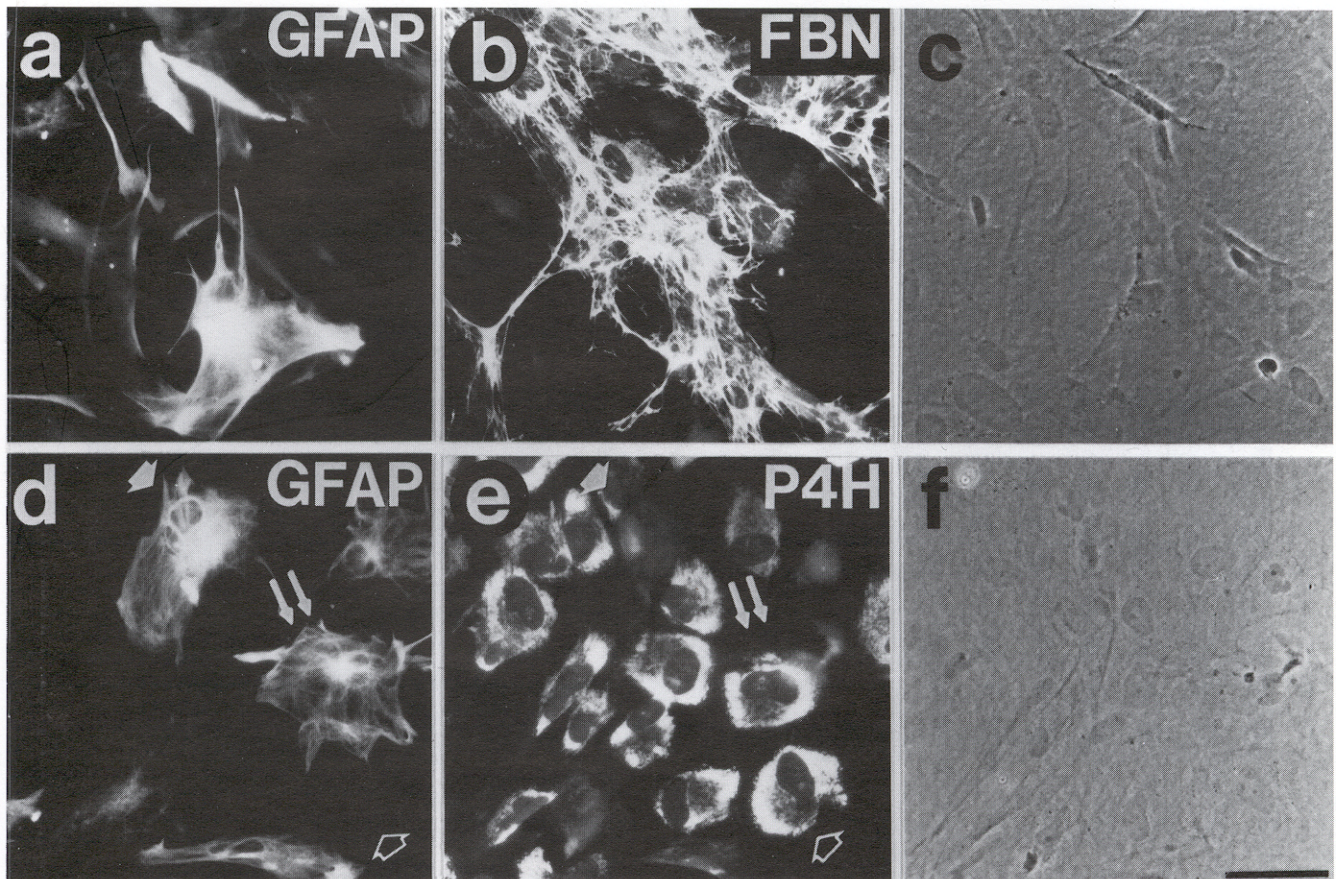
Figure 4 Immunofluorescent staining of putative astrocyte cultures for fibroblast antigens. Rostral human fetal CNS cultures were serially passaged twice and maintained in DF-FBS. Cells were analyzed by phase contrast microscopy (c,f) and two color immunofluorescence for GFAP (a,d) and fibronectin (FBN) (b) or prolyl-4-hydroxylase (P4H) (e). Closed arrowhead indicates the corresponding location of a GFAP-positive cell in d and e; open arrowhead indicates the corresponding location of a P4H-positive cell in d and e; double arrows indicate the corresponding location of overlapping GFAP- and P4H-positive cells in d and e. Magnification bar = 50 μm.

intact and cells remained viable for 7–9 days. By day 5 post infection, cultures maintained in B16-FGF-2 prior to CMV infection had few (less than 5%) cells expressing fibronectin; thus CMV antigen occurred predominantly in GFAP-positive astrocytes. The cultures maintained in DF-FBS contained varying proportions of GFAP-positive and fibronectin-positive cells depending on passage number, and CMV antigen was distributed in both types of cells.

Expression of transfected viral gene promoters
Both CMV and HIV-1 promoters were active in human fetal astrocytes, as determined by CAT reporter gene expression after transfection of pLTR-CAT or pCMV-CAT into cells (Table 1). β-galactosidase reporter gene expression was also detected immunohistochemically in the perinuclear and cytoplasmic space of GFAP-positive cells in 4 to 8-week-old astrocyte cultures transfected with pLTR-βgal (Figure 6) or pCMV-βgal (data not shown), directly confirming viral promoter activity in astrocytes. CMV and HIV promoter activities ranged

between 3 to 47-fold higher in cultures maintained in B16-FGF-2 (Table 1). These observed differences between cultures in viral promoter activity were not due to variations of the CAT analyses. CAT reporter expression was confirmed by parallel CAT enzyme assays using [14C] chloramphenicol and CAT ELISA quantitation of cell lysates from B16-FGF-2 and DF-FBS cultures of similar *in vitro* age. By both CAT assay methods, CAT expression was at least 3-fold higher in the population of cells grown in B16-FGF-2. Throughout the transient gene expression assays, cultures were monitored by immunofluorescence for astrocyte and fibroblast markers. By the end of the transient gene expression assay, cultures maintained in B16-FGF-2 prior to transfection had few (less than 5%) cells expressing fibronectin; the remainder expressed GFAP. In contrast, cultures maintained in DF-FBS had near confluent fibronectin expression and less than 25% of cells expressing GFAP (data not shown). CMV and HIV promoter activities were negligible in cultures highly enriched for fibroblasts by serial passage in DF-FBS to nine passages (data not shown).
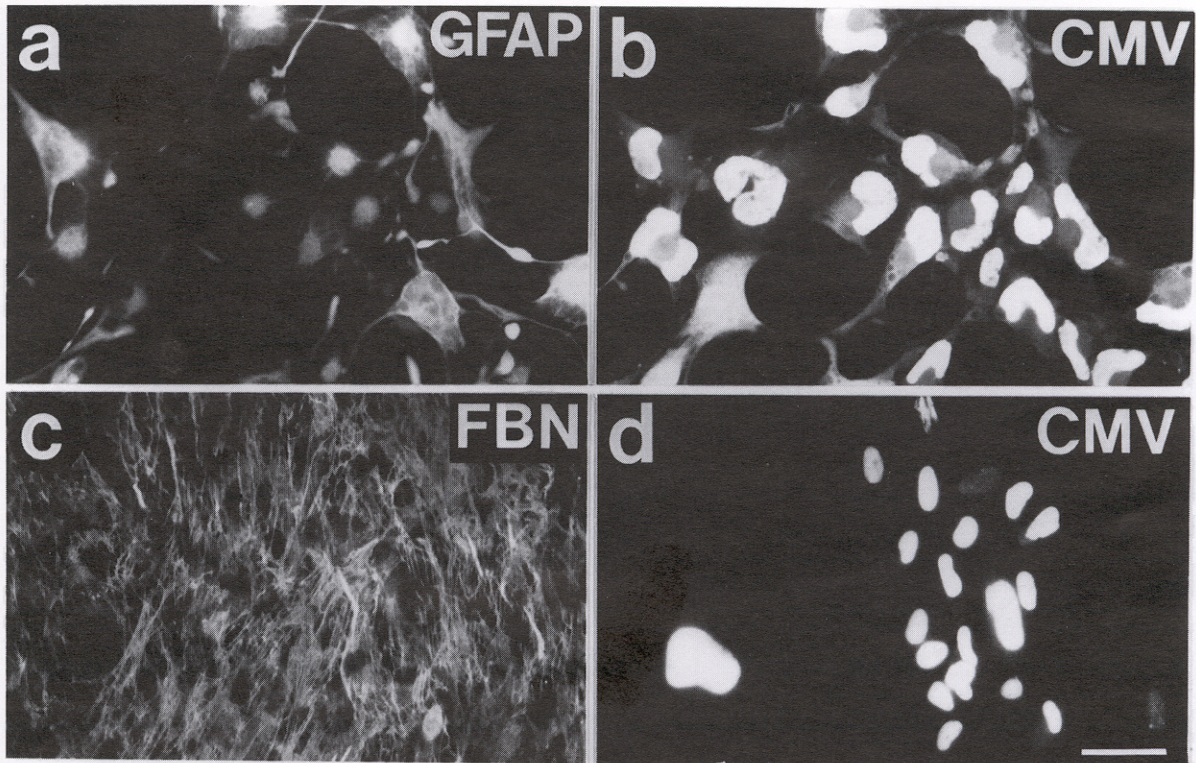
280

Figure 5 Immunofluorescent staining of CMV-infected human fetal CNS cultures maintained in B16-FGF-2 (a,b) or DF-FBS (c,d). Cultures at the seventh passage were infected with CMV strain AD169 at moi of 0.1, then analyzed 72 h post infection by two color immunofluorescence for viral and cell antigens. Cultures maintained in B16-FGF-2 had CMV antigen (b) expressed in the nuclei of GFAP-positive cells (a). Cultures maintained in DF-FBS had CMV antigen (d) expressed in the nuclei of fibronectin-positive cells (c). Similar results were obtained in four independent experiments using cultures between P2-P8. Magnification bar = 50 μm.
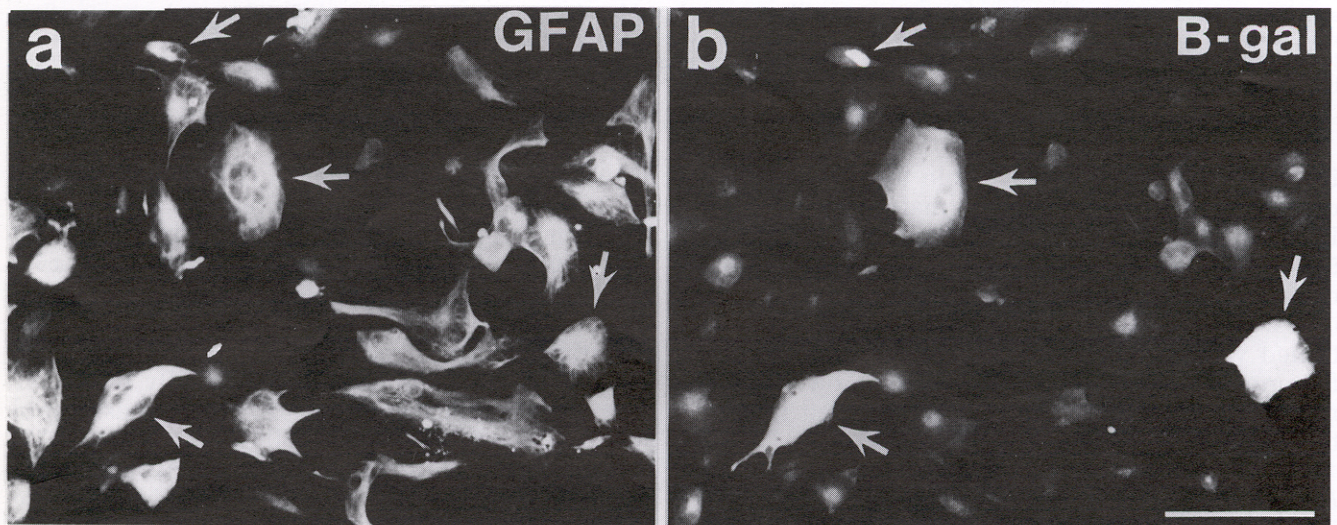

Figure 6 Two color immunofluorescent staining of human fetal astrocytes grown in B16-FGF-2 and transfected with pLTR-βgal at the fourth passage. Arrows indicate representative cells with concurrent GFAP expression (a) and β-galactosidase (B-gal) expression (b). Magnification bar = 50 μm.

Discussion

First trimester fetal human rostral CNS tissue cultured in serum-free B16 medium supplemented with FGF-2 gives rise to highly enriched if not pure populations of GFAP-positive astrocytes. Such cultures are essential in *in vitro* neurovirological studies that require highly enriched populations of astrocytes. These astrocyte cultures have minimal fibroblast contamination and maintain GFAP

Table 1 CMV and HIV-LTR promoter activities in human astrocyte cultures

Promoter construct	B16-FGF-2 culture		DF-FBS culture	
	CAT protein	CAT enzyme	CAT protein	CAT enzyme
pCMV-CAT	1631 ± 32	1.76 ± 0.33	143 ± 56	0.59 ± 0.21
pLTR-CAT	702 ± 152	1.37 ± 0.43	15 ± 12	0.22 ± 0.02
pBR322 only	0	< 0.01	0	< 0.01

Promoter activities were assayed as described in the text. CAT protein is expressed as pg per 5 µg transfected cell lysate protein. CAT enzyme specific activity is expressed as pmoles acetylated chloramphenicol per min per µg transfected cell lysate protein. Values are the average ± (range ÷ 2) of data values from two cultures maintained in B16-FGF-2 and two cultures maintained in DF-FBS. Cultures were derived from different CNS specimens, but were matched for approximate gestational age (50–60 days) and passage number (between 4 and 7).

expression when serially passaged for 1–2 months, or up to approximately eight passages (the latest passages examined). At the same time, these astrocyte cultures can support specific neurovirological assays of viral infectivity or viral gene expression.

On initial seeding and culture, CNS cell populations grown in serum-free or serum-containing culture media were very similar. Approximately 75–95% of cells were GFAP-positive, varying with the CNS tissue specimen. The GFAP-positive cell population was heterogeneous in morphology in both types of culture media, though more fibrous, processed cells predominated in cultures fed with B16-FGF-2. The heterogeneous morphology may reflect different subpopulations of GFAP-positive astrocytes, as has been demonstrated in cultures derived from human fetal brain (Elder and Major, 1988), but adult human spinal cord astrocytes show similar heterogeneous phenotypes (Whittemore et al, 1993, 1994). Few microglia are in these cultures, even at P0. This was likely due to the young gestational age of CNS tissue specimens (Aloisi et al, 1992). With as few as one to two serial passages in DF-FBS, there was extensive proliferation of fibronectin- or prolyl-4-hydroxylase-positive cells. The dense cytoplasmic and extracellular fibronectin expression, as well as the cytoplasmic prolyl hydroxylase expression, strongly suggest that these cells are fibroblasts. This fibroblast 'contamination' was clearly distinguished only after immunostaining for cell-specific antigens; morphological distinction of fibroblasts and astrocytes maintained in serum-containing medium by phase contrast microscopy alone was very difficult. The failure of fibroblasts to thrive in B16 supplemented with fibroblast growth factor suggests that some component(s) of serum is necessary for fibroblast proliferation and/or attachment.

Fibroblast contamination of fetal astrocyte cultures can confound certain types of in vitro neurovirological studies, as illustrated herein by CMV infection and viral gene promoter transient expression assays. CMV established productive infection to similar titers in fetal CNS cultures grown in serum-free or serum-containing media. These titers are comparable to those recently reported for CMV infection of primary human fetal astrocyte cultures maintained in serum-containing medium with at least 80% of adherent cells expressing GFAP (Ho et al, 1991). However, in the present study, double-label immunofluorescence of similarly-derived cultures showed CMV antigens expressed in both astrocytes (GFAP-positive cells) and fibroblasts (fibronectin-positive cells). CMV did not appear to preferentially infect astrocytes, which confounds potential studies using supernatants or lysates derived from CMV-infected astrocyte cultures that are contaminated with fibroblasts.

In contrast to CMV infectivity, viral promoter activity, measured by quantitative reporter gene expression in transfected cells, was significantly lower in fetal CNS cultures grown in DF-FBS compared to age-matched cultures grown in B16-FGF-2. Moreover, the relative expression levels of the two viral promoters showed substantial differences as well. By CAT enzyme assay, the CMV promoter was 3-fold more active and the HIV-LTR 6-fold more active in the astrocyte-enriched B-16-FGF-2 cultures as opposed to fibroblast enriched DF-FBS cultures. By CAT ELISA, the CMV promoter activity was 11-fold higher and the LTR activity was approximately 47-fold higher in the B16-FGF-2 cultures. The CAT ELISA measures only antigenically active CAT protein domains; thus it may be a less stringent but more sensitive measure of CAT gene expression than the direct measure of CAT enzyme activity. Nonetheless, the ratio of CMV promoter to HIV-1 promoter-driven CAT expression was consistently several-fold higher in the DF-FBS cultures, suggesting that CMV and HIV-1 promoter expression are differentially affected by the cell composition of fetal CNS cultures.

There are several possible explanations for the lower viral promoter activities measured in the DF-FBS cultures. The efficiency of gene transfection by cationic liposomes may be lower in fibroblasts, thus lowering the overall transfection efficiency in cultures with significant fibroblast composition. Specific promoter activities could be lower in transfected fibroblasts compared to transfected astrocytes, perhaps due to differences in cellular transcriptional factors. In non-neural cells, HIV-1 LTR

282

activity varies widely with cell type and transfection method (Barry et al, 1991). Alternatively, fibroblasts may depress gene expression in neighboring astrocytes through indirect mechanisms, perhaps metabolic or cytokine-mediated. An additional possibility is that astrocyte growth declines faster on serial passage in serum-containing medium compared to serum-free medium (Aloisi et al, 1992). In any case, the presence of fibroblasts in the transfected fetal astrocyte cultures confuses the interpretation of viral gene promoter activities measured in cell lysates derived from cell monolayers.

Of note, the lower CMV IE promoter activity observed in fibroblast enriched cultures did not correlate with lower infectious CMV titers in these cultures. In this study viral titers were determined only at the endpoint of a 5 day infection interval. This cumulative measure of viral replication may not be sufficiently sensitive to detect variations of 10-fold or less in the CMV IE promoter activity. Productive replication of CMV may be determined in part by additional promoters directing viral gene expression in the replication cycle. It is not clear whether other viral promoters also show differential expression in astrocytes versus fibroblasts; these promoters were not assayed. Given the possible cell-specific variations in transfection efficiency, internal positive controls should be used in comparative transient gene expression assays of viral promoter activities. The activity of 'fibroblast specific' promoters such as those regulating fibronectin or collagen biosynthesis would be expected to be optimal in fibroblasts. The GFAP promoter would be expected to be optimal in astrocytes. Reporter constructs using these promoters should serve as effective internal positive controls for transfection efficiency in future studies comparing differential viral promoter activities in astrocytes versus fibroblasts.

Major and Vacante (1989), in a study of the growth of the human polyomavirus JCV, demonstrated the importance of using separated cell populations from human fetal brain to investigate the host range of neurotropic viruses. Their study established that astrocytes, in addition to oligodendrocytes, would support JCV gene expression leading to virus multiplication. As in the present study, viral promoter activity was directly demonstrated as reporter gene expression in cells identifiable as astrocytes. Major and Vacante obtained a 'pure' GFAP positive cell population after high speed shaking of heterogeneous fetal CNS cell cultures containing astrocytes, oligodendrocytes, and their precursors. In the present study, a similar result was obtained by subculture of initially heterogeneous fetal CNS cell cultures in B16 medium supplemented with FGF-2, which acts as a mitogen for astrocytes (Sweetnam et al, 1991). Subculture in B16-FGF-2 selects against fibroblast proliferation and favors sustained proliferation of a highly enriched GFAP-positive cell population.

As demonstrated in the present study, the composition of in vitro glial cell populations may markedly affect the outcome and confuse the interpretation of neurovirological studies that address virus-cell-specific interactions. Astrocyte cultures serially passaged in B16-FGF-2 may prove optimal for neurovirological studies that investigate virus-astrocyte interactions or viral gene expression in astrocytes. By comparison, astrocytes cultured in serum-containing medium have a more limited useful lifetime for such studies, due to progressive overgrowth of fibroblasts and loss of GFAP-positive cells. This differential proliferation of specific cell types in primary fetal CNS cultures is more pronounced with increasing passage number and is less problematic with unpassaged cultures. However, with often unpredictable availability and viability of CNS tissue, unpassaged cultures may not provide sufficient numbers of cells to perform well-controlled studies. These data underscore the necessity of very careful cell purity determination, with multiple cellular markers, before drawing conclusions regarding cellular-restricted viral infectivity and/or gene expression in fetal human CNS cultures.

Materials and methods

Preparation and propagation of human fetal astrocyte cultures

All cultures were prepared from first trimester human fetal specimens of 46–83 days gestation. Fetal CNS tissue was obtained from the Human Embryology Laboratory, University of Washington (Seattle, WA). Procedures for procurement and use of this human fetal CNS tissue were approved and monitored by the University of Miami School of Medicine's Medical Sciences Subcommittee for the Protection of Human Subjects. The CNS tissue from each fetal specimen was processed separately and independently, as were subsequent cell cultures; there was no pooling of CNS tissue from distinct fetal specimens. Optimal dissociated cell viability was observed when the tissue was shipped in a cryopreservation medium (Kawamoto and Barrett, 1986) containing 30mM KCl, 5mM NaOH, 5mM NaH_2PO_4 (pH 7.35), 0.5 mM $MgCl_2$, 20mM Na pyruvate, 5.5mM glucose, and 175mM sorbitol. No antibiotics or antifungal agents were added to the cryopreservation medium. Effort was made to maintain the shipping temperature as close to 12°C as possible. This temperature is optimal for survival of cryopreserved human CNS tissue (Kawamoto and Barrett, 1986; TB Freeman, personal communication). This was empirically accomplished by wrapping four to five layers of paper towels around the specimen tubes before placing them on the freezer bags. Viable tissue was not obtained if the interval between harvest and plating exceeded 24 h, and no specimens were used that exceeded that time window.

Small pieces of CNS tissue were placed in cold

Leibovitz L15 medium and dissected free of spinal cord, dorsal root ganglia, meninges, blood vessels, and choroid plexus. Tissue pieces consisting primarily of rhombencephalon and mesencephalon were then transferred to serum-containing culture medium, approximately 2 ml per 500 mg tissue. Serum-containing culture medium usually consisted of Dulbecco's Modified Eagle's Medium (DMEM) plus Ham's F12 medium (F12) in equal proportions supplemented with 10% (v/v) fetal bovine serum (DF-FBS). In initial studies, cultures were also established and subcultured with DMEM plus 10% FBS or Eagle's Minimum Essential Medium (EMEM) plus 10% FBS. Culture growth and cell characteristics were the same with all three serum-containing media formulations. Thus, for experiments contrasting cultures grown in serum-containing *versus* serum-free media, adherent cells were fed and maintained in DF-FBS or serum-free medium (see below).

Tissue was mechanically dissociated by repeated trituration (up to six passages) with a 1 ml serological pipette followed by a glass Pasteur pipette. The viability of the resulting cell suspension was determined by Trypan Blue exclusion; it ranged from 30%–70% among 40 specimens. The cell suspension was then further diluted in DF-FBS and plated onto poly-L-lysine (Sigma Chemical, St Louis, MO)-coated tissue culture dishes, using either a single 100mm dish or two 60mm dishes per 500 mg CNS tissue. After 24 h, non-adherent cells were re-triturated, then re-plated onto new poly-lysine coated tissue cultures dishes; adherent cells were fed with DF-FBS. The next day all adherent cells were fed with a mixture of B16 medium (Brewer and Cotman, 1989) supplemented with 5ng ml^{-1} basic fibroblast growth factor (FGF-2) plus DF-FBS, to give a total serum concentration of 0.5% (v/v). Human recombinant FGF-2 was kindly provided by Dr Judith Abraham (Scios, Inc, Mountain View, CA). After an additional 24–48 h, all non-adherent cells were discarded; adherent cells were re-fed with the supplemented B16 medium (B16-FGF-2). The initial fetal CNS cultures were subcultured at 80–90% confluence, usually 5–10 days after seeding. Cells were harvested with 0.05% trypsin in 0.01% EDTA (GIBCO BRL, Gaithersburg, MD). Harvested cells were re-plated onto poly-L-lysine tissue culture dishes at approximately 5×10^5 cells cm^{-2}. For serial passage, cells were subcultured when confluent, approximately once per week, and passaged up to eight times. Infection or transfection studies were performed with cultures serially passaged a minimum of two or more times in order to generate sufficient numbers of cells with which to do controlled assays.

Immunohistochemical staining
Cultures were assayed at serial passages for the following cell-specific antigens: glial fibrillary acidic protein (GFAP) for astrocytes, CD68 for microglia, neuron specific enolase (NSE) for neurons, O4 for oligodendrocytes, A2B5 for bipotential progenitors capable of differentiating into astrocytes (Kennedy and Fok-Seang, 1986) and fibronectin or prolyl-4-hydroxylase for fibroblast-type cells. Cells were cultured on poly-L-lysine-coated glass cover slips in 35mm wells. To label antigens GFAP, NSE, CD68, fibronectin, and prolyl-4-hydroxylase, cells were fixed for 7–10 min with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2, at room temperature, then treated with 0.2% (v/v) Triton X-100 in phosphate buffered saline (PBS) for 5 min. To label cell surface antigens including A2B5 and O4, cells were fixed for 10 min with 10% buffered formalin with 5% (w/v) sucrose. Indirect immunofluorescence was performed with the following primary antibodies: rabbit polyclonal anti-GFAP, mouse monoclonal IgG anti-CD68 (clone EBM-11), mouse monoclonal IgG anti-prolyl-4-hydroxylase (all DAKO Corp, Carpinteria, CA); mouse monoclonal IgG anti-human fibronectin (clone 3E3, Boehringer Mannheim, Indianapolis, IN); rabbit polyclonal anti-human fibronectin (GIBCO BRL, Gaithersburg, MD); mouse monoclonal IgM anti-A2B5 (American Type Culture Collection, Rockville, MD, clone 105, CRL 1520); mouse monoclonal IgG anti-O4 (kindly provided by Dr Melitta Schachner, Swiss Federal Institute of Technology, Zurich, Switzerland); mouse monoclonal IgG anti-NSE (Polysciences Inc, Warrington, PA). Secondary antibodies included goat anti-rabbit IgG conjugated to rhodamine, and goat anti-mouse IgG or goat anti-mouse IgM conjugated to fluorescein (FITC) (Boehringer Mannheim, Indianapolis, IN).

CMV infection of fetal astrocytes cultures
Cultures growing in six-well tissue culture plates were washed with PBS, then inoculated with 0.1 pfu cell^{-1} of CMV strain AD 169 (hereafter referred to as CMV) grown and titered in human lung fibroblast (MRC-5) cultures. Inoculum was diluted and adsorbed in DF medium without serum for 2 h at 37°C. Inoculum medium was then replaced with 2ml DF-5% FBS per 10^6 cells. Cultures were monitored for cytopathic effect by phase contrast microscopy and immunohistochemistry for intracellular CMV antigen using mouse monoclonal IgG antibodies to CMV nuclear immediate early and early antigens (DAKO, Carpinteria, CA). Mock-infected cultures were also immunohistochemically assayed for fibronectin and GFAP expression during the infection time course. Infected culture supernatants and cell lysates were harvested for virus titration 5 days post infection. Lysates were prepared from infected monolayers by three cycles of freeze-thaw in 2ml phosphate buffered saline (PBS) per 10^6 cells. Infectious CMV was titered by plaque assay on MRC-5 monolayers.

Transient gene expression assays

Two viral promoters were assayed in these fetal astrocyte cultures: the HIV-1 long terminal repeat (LTR) and the CMV immediate early (IE) promoter. Recombinant LTR plasmids containing the cloned HIV-1 LTR were constructed by replacing the SV40 promoter in the pSV2-CAT plasmid with the HIV-1 LTR containing both the 3′ untranslated regions (U3) and the repeat sequences (R) Kashanchi and Wood, 1989). This generated a plasmid with the HIV-1 LTR linked to CAT (pLTR-CAT). The CAT gene in pLTR-CAT was replaced by the β-galactosidase gene to generate an additional LTR plasmid, pLTR-βgal. Plasmid pCMV-CAT was constructed by replacing the LTR of the pLTR-CAT construct (Chang *et al*, 1993) with the CMV IE promoter (kindly provided by Dr Lung-Ji Chang, University of Alberta, Alberta, Canada). Plasmid DNA was transfected into cells using cationic liposomes (Lipofectamine, GIBCO BRL, Gaithersburg, MD) mixed with 1–2 µg plasmid DNA per 10^6 cells. Control cultures were transfected with 1–2 µg carrier plasmid pBR322 DNA. DNA-liposome mixtures were incubated with cultures for 8–14 h in serum-free medium, then cultures were maintained in DF-FBS. Transfected cultures were harvested 72 h after transfection for all reporter gene assays. Lysates of infected cells were prepared by three cycles of freeze-thaw followed by incubation at 65°C for 5 min. Lysate protein was quantitated by the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. CAT protein in lysates was quantitated with a commercial ELISA assay (Boehringer Mannheim, Indianapolis, IN). CAT reporter gene expression was also verified by CAT enzyme assay, measured as conversion of [^{14}C]chloramphenicol (59.7 mCi/mmole, DuPont NEN, Wilmington, DE) to acetylated derivatives (Kingston and Sheen, 1993). The CAT enzyme assays used 10 or 15 ug aliquots of lysate protein incubated for 3 h at 37°C. β-galactosidase expression in transfected cultures was assayed immuno-histochemically following fixation with paraformaldehyde/Triton X-100 at 72 h post transfection. The primary antibody was a mouse monoclonal IgG anti-β-galactosidase (Boehringer Mannheim, Indianapolis, IN). Mock-transfected cultures were also monitored immunohistochemically for fibronectin and GFAP expression during the transient gene expression assay time course.

References

Aloisi F, Borsellino G, Samoggia P, Testa U, Chelucci C, Russo G, Peschle C, Levi G (1992). Astrocyte cultures from human embryonic brain: characterization and modulation of surface molecules by inflammatory cytokines. *J Neurosci Res* **32**: 494–506.

Barry PA, Pratt-Lowe E, Unger RE, Luciw PA (1991) Cellular factors regulate transactivation of human immunodeficiency virus type 1. *J Virol* **65**: 1391–1399.

Blumberg BM, Gelbard HA, Epstein LG (1994). HIV-1 infection of the developing nervous system: central role of astrocytes in pathogenesis. *Virus Res* **32**: 253–267.

Brack-Werner R, Kleinschmidt A, Ludvigsen A, Mellert W, Neumann M, Herrmann R, Khim MC, Burny A, Muller-Lantzsch N, Stavrou D (1992). Infection of human brain cells by HIV-1: restricted production in chronically infected human glial cell lines. *AIDS* **6**: 273–285.

Brewer GJ, Cotman CW (1989). Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. *Brain Res* **494**: 65–74.

Chang L-J, McNulty E, Martin M (1993). Human immunodeficiency viruses containing heterologous enhancer/promoters are replication competent and exhibit different lymphocyte tropisms. *J Virol* **67**: 743–752.

Chiodi F, Fuerstenberg S, Gidlund M, Asjo B, Fenyo EM (1987). Infection of brain-derived cells with the human immunodeficiency virus. *J Virol* **61**: 1244–1247.

Dewhurst S, Sakai K, Bresser J, Stevenson M, Evinger-Hodges MJ, Volsky DJ (1987). Persistent productive infection of human glial cells by human immunodeficiency virus (HIV) and by infectious molecular clones of HIV. *J Virol* **61**: 3774–3782.

Elder GA, Major EO (1988). Early appearance of type II astrocytes in developing human fetal brain. *Dev Brain Res* **42**: 146–150.

Ho W-Z, Song L, Douglas SD (1991). Human cytomegalovirus infection and transactivation of HIV-1 LTR in human brain-derived cells. *J Acquir Immune Defic Syndr* **4**: 1098–1106.

Janin A, Konttinen YT, Gronblad M, Karchunen P, Gosset D, Malmstrom M (1990). Fibroblast markers in labial salivary gland biopsies in progressive systemic sclerosis *Clin Exp Rheumatol* **8**: 237–242.

Jault FM, Spector SA, Spector DH (1994). The effects of cytomegalovirus on human immunodeficiency virus replication in brain-derived cells correlate with permissiveness of the cells for each virus. *J Virol* **68**: 959–973.

Kashanchi F, Wood C (1989). Human immunodeficiency viral long terminal repeat is functional and can be

transactivated in *E Coli. Proc Natl Acad Sci USA* **86**: 2157–2161.

Kawamoto JC, Barrett JN (1986). Cryopreservation of primary neurons for tissue culture. *Brain Res* **384**: 84–93.

Kennedy PGE, Fok-Seang J (1986). Studies on the development, antigenic phenotype and function of human glial cells in tissue culture. *Brain* **109**: 1261–1277.

Kennedy PGE, Lisak RP, Raff MC (1980). Cell type-specific markers for human glial and neuronal cells in culture. *Lab Invest*, **43**: 342–351.

Kingston RE, Sheen J (1993). Reporter system using chloramphenicol acetyl transferase. In: Ausubel FM, Brent R, Kingston RE *et al* (eds). *Current Protocols in Molecular Biology*. J Wiley & Sons: New York, Volume I, Suppl 14, pp 9.6.3–9.6.5.

Kohleisen B, Neumann M, Herrmann R, Brack-Werner R, Krohn KJE, Ovid V, Ranki A, Erfle V (1992). Cellular localization of Nef expressed in persistently HIV-1-infected low-producer astrocytes. *AIDS* **6**: 1427–1436.

Lee SC, Liu W, Brosnan CF, Dickson DW (1992). Characterization of primary human fetal dissociated central nervous system cultures with an emphasis on microglia. *Lab Invest* **67**: 465–476.

Major EO, Vacante DA (1989). Human fetal astrocytes in culture support the growth of the neurotropic human polyomavirus, JCV. *J Neuropathol Exp Neurol* **48**:425–436.

McCarthy M, Fedosyeva L, Wood C (1994). Regulation of HIV promoter-directed gene expression in primary human astrocytes. *Neurology* **44**: A396.

McCarthy M, Fedoseyeva L, He J, Wood C (1995). HHV-6 in primary human astrocytes: productive infection and enhancement of HIV promoter expression. *Neurology*

45: A438.

Sharpless N, Gilbert D, Vandercam B, Zhou JM, Verdin E, Ronnett G, Friedman E, Dubois-Dalcq M (1992). The restricted nature of HIV-1 tropism for cultured neural cells. *Virology* **191**: 813–825.

Shein HM (1965). Propagation of human fetal spongioblasts and astrocytes in dispersed cell cultures. *Exp Cell Res* **40**: 554–569.

Sweetnam PM, Sanon HR, White LA, Brass BJ, Jaye M, Whittemore SR (1991). Differential effects of acidic and basic fibroblast growth factor on spinal cord cholinergic, GABAergic, and glutamatergic neurons. *J Neurochem* **57**: 237–249.

Tornatore C, Nath A, Amemiya K, Major EO (1991). Persistent human immunodeficency virus type 1 infection in human fetal glial cells reactivated by T-cell factor(s) or by the cytokines tumour necrosis factor alpha and interleukin-1 beta. *J Virol* **65**: 6094–6100.

Tornatore C, Meyers K, Atwood W, Conant K, Major E (1994). Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* **68**: 93–102.

Watkins BA, Dorn HH, Kelly WB, Armstrong RC, Potts BJ, Michaels F, Kufta CV, Dubois-Dalcq M (1990). Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science* **249**: 549–553.

Whittemore SR. Sanon HR, Wood PM (1993). Concurrent isolation and characterization of oligodendrocytes, microglia, and astrocytes from adult human spinal cord. *Int J Dev Neurosci* **11**: 755–764.

Whittemore SR, Neary JT, Kleitman N, Sanon HR, Benigno A, Donahue RP, Norenberg MD (1994). Isolation and characterization of conditionally immortalized astrocyte cell lines derived from adult human spinal cord. *Glia* **10**: 211–226.