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Evaluation of the role of cytokine activation in the multiplication of JC virus (JCV) in human fetal glial cells

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The human polyomavirus, JCV, is the etiologic agent of the fatal central nervous system demyelinating disease, progressive multifocal leukoencephalopathy. Progressive multifocal leukoencephalopathy occurs most frequently in patients with underlying immunosuppressive disorders and is the direct result of virus multiplication in oligodendrocytes, the myelin producing cell in the central nervous system. In this report we test the ability of cellular activation signals to modulate expression of the JCV genome in either transfected or infected human fetal glial cells. In addition, we analyze the binding of nuclear proteins isolated from untreated and cytokine treated human fetal glial cells to transcription factor binding sites in the JCV regulatory region. In contrast to the effects of cellular activation on the expression of the HIV-1 promoter in these cells, none of the cellular activators tested increased expression of JCV. The cytokine, TNF-α, increased binding of NFκB (p50/p65) to a JC NFκB site but did not modulate the binding of nuclear proteins to the overlapping NF-1/AP1 region of the JCV enhancer. When taken together these results suggest that the response of JCV to cellular activation signals may be fundamentally different from the response of HIV-1 to these signals in human fetal glial cells and that the JC NFκB site may not be required for JCV gene expression or multiplication *in vivo*.

Keywords: progressive multifocal leukoencephalopathy; transcription factors; NFκB

Introduction

JC virus belongs to the family papovaviridae and the genus polyomavirinae (Walker and Frisque, 1986; Major *et al*, 1992). Polyomaviruses are found in a wide variety of other species and are ubiquitous in their respective natural hosts. Seroepidemiological studies have indicated that greater than 70% of the human population worldwide have been exposed to JC virus (Walker and Padgett, 1983). The association of JC virus with a once rare human demyelinating disease known as progressive multifocal leukoencephalopathy (PML) was firmly established two decades ago with the cultivation of the virus from an afflicted individual whose initials were JC (Padgett *et al*, 1971). The incidence of PML has risen dramatically as a result of the AIDS pandemic. Approximately 2–4% of AIDS patients will develop PML (Berger *et al*, 1987; Kure *et al*, 1991).

In vivo, JCV has been detected in oligodendrocytes, astrocytes, in lymphoid tissues, and in peripheral blood lymphocytes of PML patients (Mazlo and Tariska, 1980; Richardson and Webster, 1983; Weiner *et al*, 1973; Tornatore *et al*, 1992; Houff *et al*, 1988; Schneider and Dorries, 1993; Dorries *et al*, 1994). *In vitro*, JCV infects glial cells derived from human fetal brain and, to a limited extent, several B lymphocyte cell lines (Atwood *et al*, 1992; Major and Vacante, 1989). This highly restricted cell type specificity is thought to be due to tissue specific transcription factors that interact with the regulatory region of JCV, cellular factors that interact with the JCV T antigen, or both (Feigenbaum *et al*, 1987; Ahmed *et al*, 1990a,b; Tada and Khalili, 1992; Ranganathan and Khalili, 1993; Chowdhury *et al*, 1990; Wegner *et al*, 1993; Renner *et al*, Kumar *et al*, 1994; Henson, 1994;

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Major *et al*, 1990; Atwood *et al*, 1992; Tamura *et al*, 1990; Tamura *et al*, 1988). Studies of JCV regulation have mainly focused on the 98 base pair repeat region of the JCV genome that has been shown to contain binding sites for several transcription factors, including the nuclear factor-1 (NF-1) and the activator protein-1 (AP-1/c-jun) (Tamura *et al*, 1988; Amemiya *et al*, 1989, 1992). In addition to transcription factor binding sites in this region we and others have described the presence of a potential nuclear factor-κ (NFκB) binding site outside the JCV 98 base pair repeats (Major *et al*, 1990; Ranganathan and Khalili, 1993). Recently, transcription from a JCV promoter construct containing an NFκB site was found to be increased by treatment of human glioblastoma cells with the phorbol ester, PMA (Ranganathan and Khalili, 1993).

In this report we examine the effects of cellular activation on the multiplication of JCV in infected and transfected human fetal glial cells. In addition we analyze the binding of nuclear proteins isolated from untreated and TNF-α treated glial cells to oligonucleotides containing either the JCV NF-1/AP1 site or the JC NFκB site. Using a panel of cellular activating agents we were unable to show activation of a reporter gene under the control of the JCV regulatory region. This correlated with gel shift assays which showed no differences in the binding of nuclear proteins to the NF-1/AP1 oligonucleotide from untreated or TNF-α treated glial cells. In contrast, treatment of the glial cells with TNF-α increased the binding of NFκB (p50/p65) to an oligonucleotide containing the putative JC NFκB site. Since our CAT construct did not include the JC NFκB site we infected glial cells with JC virus to assess the contribution of this site to the multiplication of JCV in these cells. Treatment of the infected cells with a known NFκB inducer, TNF-α, did not result in an increase in the percentage of cells expressing T or V antigens or in the percentage of *in situ* hybridization positive cells. This is in contrast to the effects of TNF-α, IL-1β, and PMA on multiplication of HIV-1 in these cells (Atwood *et al*, 1994; Tornatore *et al*, 1994; Conant *et al*, 1994).

Our results suggest that the response of JCV to cellular activation signals may be fundamentally different from the response of HIV-1 to these signals in human fetal glial cells and that the JC NFκB site may not be required for JCV gene expression and multiplication *in vivo*.

Results

JC virus reporter gene expression in response to cellular activation

A JC virus construct (pM1CAT) containing the JCV regulatory region cloned upstream of a chloramphenicol acetyltransferase gene (CAT) was transfected into human fetal glial cells as described in materials and methods. The putative NFκB binding site is outside of the regulatory region of JCV and is therefore not included in this construct. Following transfection the cells were cultured for 48 h in media alone or with media containing either TNF-α, Il-1β, Il-2, Il-6, GM-CSF, or a mixture of PMA and the calcium ionophore A23187. Treatment of the transfected glial cells with these cytokines or PMA and calcium ionophore did not increase the expression of CAT from the JCV promoter (Figure 1). In replicate experiments, TNF-α inhibited JCV promoter activity by 52.9%, Il-1β inhibited by 72.2%, Il-6 inhibited by 53.0%, Il-2 inhibited by 48.1%, GM-CSF inhibited by 51.7%, and the combination of PMA and calcium ionophore (A23187) inhibited by 90.0%. The effect observed is specific to the JCV construct as under identical experimental conditions several of these reagents routinely increase the expression of CAT under the control of an HIV-1 promoter (Atwood *et al*, 1994).

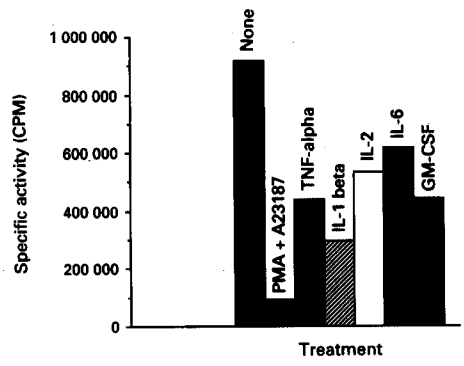

Figure 1 Expression of pM1CAT in transiently transfected human fetal glial cells in the presence and absence of cellular activating agents. Human fetal glial cells were transfected with pM1CAT as described in materials and methods and then cultured for 48 h in absence (none) or presence of cellular activating agents as indicated. Specific activity is expressed as the change in counts per min (CPM) per mg of protein per h.

JC virus reporter gene expression in response to cocultivation with B and T lymphocyte cell lines

It is possible that cell to cell signaling or soluble factors produced by cells other than the ones tested here may lead to increases in JCV gene expression. Since JCV infection is associated with lymphocytes in peripheral blood, in bone marrow, and in brain parenchyma, we examined JCV promoter activity in glial cells in the presence and absence of cocultivation with B and T lymphocyte cell lines (Atwood *et al*, 1992; Houff *et al*, 1988). Neither the Namalwa B cell line nor the A3.01 T cell line when cocultured in direct contact with transfected glial cells were capable of providing a signal that would activate expression of the JCV promoter (Figure 2). Cocultivation with either Namalwa cells or A3.01

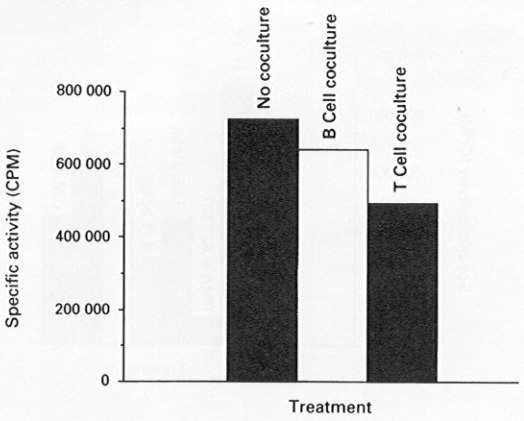

Figure 2 Expression of pM1CAT in transiently transfected human fetal glial cells in the presence or absence of cocultivation with B (Namalwa) and T (A3.01) lymphocyte cell lines. Specific activity is expressed as the change in counts per min (CPM) per h per mg of protein.

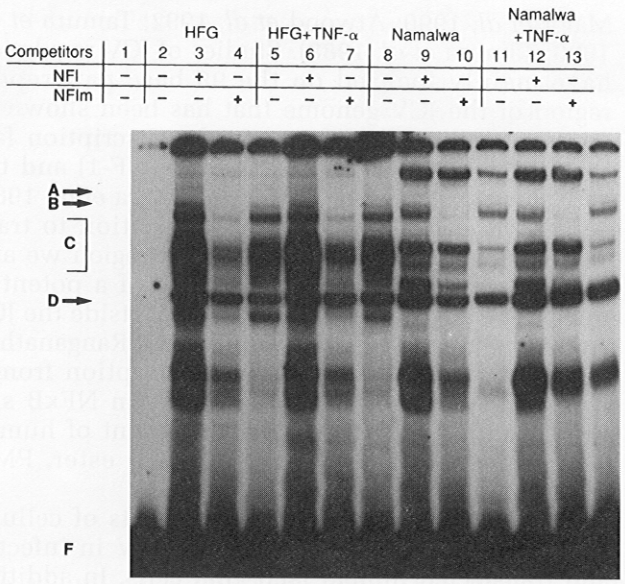

Figure 3 Gel shift analysis of the binding of nuclear proteins from untreated and TNF-α treated HFG and Namalwa cells to an oligonucleotide containing the overlapping NF1/AP1 binding site. The presence of competitor oligonucleotides are indicated at the left and by a (+) over the lanes to which they were added. Lane 1 is probe alone. F indicates the migration of the free probe in the absence of added nuclear extract. Lanes 2–4 are the probe incubated with nuclear extracts from untreated HFG cells. Lanes 5–7 are the probe incubated with nuclear extracts from TNF-α treated HFG cells. Lanes 8–10 are the probe incubated with nuclear extracts from Namalwa cells. Lanes 11–13 are the probe incubated with nuclear extracts from Namalwa cells treated with TNF-α. Lanes 3, 6, 9, and 12 have an excess of unlabeled homologous competitor in the DNA binding reaction. Lanes 4, 7, 10, and 13 have an excess of unlabeled mutant competitor in the DNA binding reaction. A, B, C, and D indicate the positions of specific gel shifted bands. Note that only the B complex is seen when extracts from Namalwa cells are reacted with the probe. The migration and intensity of the bands (A–D) is unaffected by TNF-α treatment.

cells resulted in a decrease in activity of the JCV promoter. This effect is specific to the JCV construct as identical experiments using a construct containing the HIV-1 promoter result in an increase in HIV-1 gene expression and multiplication (Tornatore *et al*, 1991).

Effect of TNF-α on the binding of transcription factors to the overlapping NF-1/AP1 site in the JCV regulatory region

Since the overlapping NF-1/AP-1 binding site in the JCV enhancer is critical to both transcription and replication of the JCV genome we examined nuclear protein binding to this region in the context of TNF-α stimulation. Several specific gel shifted complexes (Figure 3, A–D) were formed when nuclear proteins from untreated (Figure 3, lanes 2–4) and TNF-α treated (Figure 3, lanes 5–7) human fetal glial cells were interacted with the probe. These complexes are specific as they were completely competed for by an excess of unlabeled homologous probe (Figure 3, lanes 3 and 6) but not by an excess of unlabeled mutant probe (Figure 3, lanes 4 and 7). When the probe was reacted with nuclear extracts from glial cells that had been treated with TNF-α no discernible differences in the specific binding of any of the complexes were observed (Figure 3, compare lanes 2–4 with lanes 5–7). Since JCV is capable of infecting several B cell lines we also examined nuclear protein binding to our probe from untreated and TNF-α treated B cells (Namalwa). Only the B complex was formed when nuclear extracts from these cells were interacted with our probe (Figure 3, lanes 8–10). Again, no differences were seen when the nuclear extracts were from Namalwa cells treated with TNF-α (Figure 3, lanes 11–13).

Effect of TNF-α stimulation on the binding of nuclear proteins to the JC NFκB site

To begin to assess the role of the putative NFκB site in the multiplication of JCV we tested whether or not nuclear proteins isolated from primary human fetal glial cells would bind to an oligonucleotide containing this site. Nuclear proteins isolated from human fetal glial cells formed two detectable gel shifted complexes when reacted with an oligonucleotide containing the JCκB site (Figure 4, lanes 2–5, bold and light arrows). The complexes are specific as they were completely competed for an excess of unlabeled homologous probe (Figure 4, lane 3) but not by an excess of unlabeled mutant probe (Figure 4, lane 4). Three gel shifted complexes were evident when the probe was interacted with extracts from glial cells that had been treated with TNF-α (Figure 4, lanes 6–9, bold, light, and dashed arrows). The complexes are specific as they were completely competed for an excess of unlabeled mutant probe (Figure 4, lane 7) but not by an excess

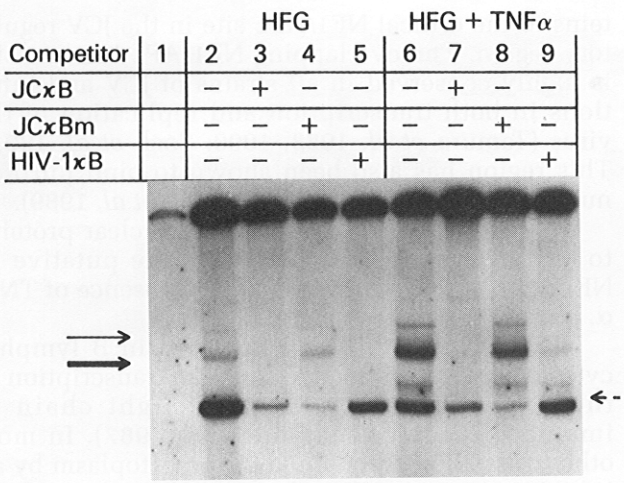

Figure 4 Gel shift analysis of the binding of nuclear proteins from untreated and TNF-α treated HFG cells to an oligonucleotide containing the JC NFκB site. The presence of competitor oligonucleotides are indicated at the left and by a (+) over the lanes to which they were added. Lane 1 is probe alone. Note that the migration of free probe is not shown in this figure. Lanes 2–5 are the probe incubated with nuclear extract from untreated HFG cells. Lanes 6-9 are the probe incubated with nuclear extracts from TNF-α treated HFG cells. The arrows indicate the position of specific gel shifted bands. All of the specific bands are increased several-fold in extracts from TNF-α treated HFG cells.

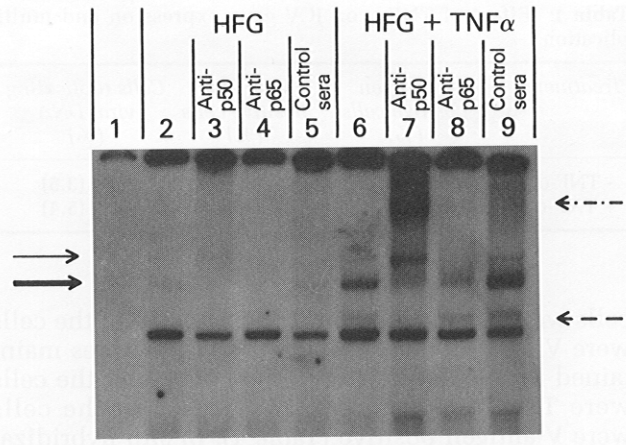

Figure 5 Supershift analysis of the specific JC NFκB binding proteins in HFG cells and HFG cells treated with TNF-α. Lane 1 is probe alone. Note that the migration of free probe is not shown in this figure. Lanes 2–5 are the probe incubated with nuclear extract from untreated HFG cells. Lanes 6–9 are the probe incubated with nuclear extract from TNF-α treated HFG cells. Antisera to the p50 and p65 subunits of NFκB or control antisera were added to the DNA binding reactions as described in materials and methods. Lanes 2 and 6 are the DNA binding reaction to which no antisera was added. Lanes 3 and 7 are the DNA binding reaction to which anti-p50 sera was added. Lanes 4 and 8 are the DNA binding reaction to which anti-p65 sera was added. Lanes 5 and 9 are the DNA binding reaction to which control antisera was added. The arrows indicate the positions of specific gel shifted complexes which are supershifted or abolished by incubation with anti p50 and p65 antisera respectively. The bold arrow indicates the position of a faint band in untreated lanes and an induced band in TNF-α treated lanes which is affected by both anti-p50 and anti-p65 sera. The dashed-dotted arrow indicates the new position of the anti-p50 supershifted band. The light-solid arrow indicates the position of a specific band which is only affected by anti-p65 sera. The dashed arrow indicates the position of a specific band which is only affected by the anti-p50 sera.

of unlabeled mutant probe (Figure 4, Lane 8). Binding to the JC NFκB probe was induced several fold by TNF-α treatment (Figure 4, compare lanes 2–5 with 6–8). An excess of unlabeled HIV-1 κB competitor competed for all the specific complexes from either uninduced or induced glial cells (Figure 4, lanes 5 and 9).

Identification of NFκB subunits present in each of the specific gel shifted complexes with subunit specific antisera

Nuclear proteins from untreated and TNF-α treated glial cells were reacted with the JC NFκB probe in the presence and absence of antisera to the p50 and p65 subunits of NFκB. Antisera to an unrelated antigen, galactocerebroside, was used as a negative control in this experiment. Anti-p50 antisera supershifted two of the specific bands (Figure 5, compare lanes 2 and 6 with lanes 3 and 7). The dashed-dotted arrow indicates the new position of the anti-p50 supershifted band. Anti-p65 antisera abolished two of the specific bands (Figure 5, compare lanes 2 and 6 with lanes 4 and 8). The control anti-sera had no effect on the migration or the formation of any of the bands (Figure 5, lanes 5 and 9). The bold arrow indicates the position of a band that is affected by both the p50 and p65 specific antisera which indicates that both p50 and p65 subunits are present in the complex. The dashed arrow indicates the position of a band that is only affected by p50 antisera which indicates that p50 but not p65 is present in that complex. The solid-light arrow indicates the position of a band that is only affected by the p65 antisera which indicates that p65 but not p50 is present in that complex.

Effect of TNF-α stimulation on the multiplication of JC virus in infected human fetal glial cells

Since our molecular construct did not contain the putative JC NFκB binding site we assessed the effects of TNF-α stimulation on JC virus infected glial cells. The MAD-4 strain of virus used in this experiment has been sequenced and found to contain the putative κB site (our unpublished results). Following infection of cell monolayers with virus the cells were maintained in media alone or in media containing TNF-α (1000 U ml^{-1}). At 5 days post infection the percentages of T and V antigen positive cells as well as the percentage of *in situ* hybridization positive cells were determined. In cultures maintained without TNF-α, 46.9% of the

Table 1 Effect of TNF-α on JCV gene expression and multiplication

Treatment	*T antigen positive cells (%)*	*V antigen positive cells (%)*	*Cells replicating viral DNA (%)*
- TNF-α	46.9 (18.5)	26.1 (5.8)	15.9 (3.5)
+ TNF-α	47.1 (15.1)	21.7 (4.2)	13.4 (5.4)

cells were T antigen positive, and 26.1% of the cells were V antigen positive (Table 1). In cultures maintained in the presence of TNF-α, 47.1% of the cells were T antigen positive, and 21.7% of the cells were V antigen positive (Table 1). *In situ* hybridization with a biotinylated JCV DNA probe detected 15.9% positive cells in the untreated cultures and 13.4% positive cells in the TNF-α treated cultures (Table 1). Note that we have previously determined that the sensitivity of *in situ* hybridization with biotinylated JCV DNA probes is such that several hundred copies of the JCV genome need to be present in order to generate a positive signal. Representative T antigen, V antigen, and *in situ* hybridization positive cells are shown in Figure 6, panels A, B, and C respectively. Standard error of the mean is indicated by the numbers in parentheses.

Discussion

JC virus is an extremely successful human pathogen, infecting greater than 70% of the population worldwide. Part of this success no doubt comes from the ability of JCV to establish life long persistent or latent infections in its host. Periodic reactivation of the virus and its excretion in urine probably contribute to the high carrier rate of virus in the population. Intensive research on JCV began nearly two decades ago when the virus was associated with the rare but fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML). Despite this, little is known about the mechanisms of virus transmission, virus latency, or the signals that lead to its reactivation.

Since a number of inflammatory cytokines are increased in acquired immune deficiency syndrome (AIDS) patients, in particular in CNS tissue, we sought to determine what role, if any, cytokines play in the life of JCV *in vivo* (Wesselingh *et al*, 1993). Using a panel of cellular activating agents that included TNF-α, IL-1β, IL-2, IL-6, GM-CSF, PMA in combination with the calcium ionophore A23187, and cocultivation with B cells and T cells, we were unable to increase expression of a reporter gene under the control of the JCV regulatory region. In fact, these treatments all led to lower expression of the reporter gene in these cells. Consistent with this lack of induction is the fact that we could not detect any modulation of binding of nuclear proteins to the critical NF1/AP1 site in the JCV regulatory region. The overlapping NF1/AP1 binding site is highly conserved in all strains of JCV and functions in both transcription and replication of the virus (Tamura *et al*, 1988, 1990; Sock *et al*, 1991). This region has also been shown to bind purified nuclear factor-1 and c-jun (Amemiya *et al*, 1989).

We next investigated binding of nuclear proteins to an oligonucleotide containing the putative JC NFκB site both in the presence and absence of TNF-α, a known inducer of NFκB.

NFκB is constitutively expressed in B lymphocytes where it functions to increase transcription of the gene that codes for the κ light chain of immunoglobulins (Lenardo *et al*, 1987). In most other cells NFκB is retained in the cytoplasm by an inhibitor protein referred to as inhibitor κB (IκB) (Bours *et al*, 1992b: Brown *et al*, 1993). Activation of cells by a variety of signals, including TNF-α and Il-1β, result in the dissociation of NFκB from IκB and translocation of NFκB to the nucleus. In the nucleus NFκB activates the expression of several viral and cellular genes (Kawakami *et al*, 1988; Lenardo and Baltimore, 1989; Lenardo *et al*, 1989). The prototypical and most abundant form of NFκB is a heterodimer composed of p50 and p65 subunits. Recently, however, several other homodimeric and heterodimeric forms of NFκB have been described (Molitor *et al*, 1990; Bours *et al*, 1992b; Baeuerle, 1991; Mercurio *et al*, 1993; Bours *et al*, 1992a). These include p50/p50 homodimers and p65/p65 homodimers (Ganchi *et al*, 1993).

In contrast to our results using the NF1/AP1 probe, TNF-α treatment of glial cells led to a several fold induction of binding of nuclear proteins to an oligonucleotide probe containing the JC NFκB site. Supershift analysis with NFκB specific antisera was used to confirm that the gel shifted bands were indeed due to the binding of NFκB p50 and p65 subunits. The predominant band is due to the binding of p50/p65 heterodimers, and the other minor bands are most likely due to the binding of p50/p50 and p65/p65 homodimers. TheHIV-1 NFκB site effectively competed for all three bands in both induced and uninduced cells. These results are consistent with our previous report of TNF-α induction of NFκB binding to an oligonucleotide containing an HIV-1 κB binding site (Atwood *et al*, 1994).

Since our molecular construct did not contain a κB site we sought to test the contribution of this site by infecting glial cells with virus. We measured early and late gene expression and virus multiplication at 5 days post infection. This time was chosen as it would allow us simultaneously to measure the effects of TNF-α at all stages of the JC virus life cycle. Treatment of the cultures with TNF-α did not increase either the percentage of cells expressing the early viral protein, T antigen, or the percentage of cells expressing the late viral virion protein, VP1. Similarly, the percentage of *in situ* hybridization

positive cells was not increased by TNF-α treatment of the infected cells. The sensitivity of our DNA:DNA *in situ* hybridization with biotinylated probes is such that a minimum of 200 copies of viral DNA must be present in a cell in order to be detected (Aksamit *et al*, 1985). This method therefore only detects a high copy number of viral DNA which can only occur if the DNA is being replicated in the cells.

These data are consistent with our transient transfection assays in that TNF-α actually reduced virus gene expression. It is possible that the negative effects of these reagents is due to toxicity of the reagents for the cells. We feel, however, that this is unlikely as these same reagents increase the expression and multiplication of HIV-1 in these same cells (Atwood *et al*, 1994; Tornatore *et al*, 1991). Also, several of these cytokines, including TNF-α and Il-6, are actually produced by our glial cell cultures (Vitkovic *et al*, 1991).

There are several possibilities that could account for the lack of increased JCV gene expression by TNF-α despite the induction of binding of NFκB to the JC NFκB oligonucleotide. One possibility is that binding of a transcription factor to an isolated binding site on an oligonucleotide may not reflect what actually occurs *in vivo*. Gel shift assays with oligonucleotides that include surrounding JCV DNA sequences as well as *in vivo* footprinting experiments will need to be done to determine whether NFκB binds to this region *in vivo*. Another possibility is that the binding site for NFκB is located in the early coding region for the large T protein and binding here could be inhibitory to T protein synthesis. We are currently examining the effects of TNF-α on both early and late mRNA synthesis in JCV infected cells to assess this possibility. Also, we are investigating other types of signalling pathways that may lead to increased JCV expression in cells derived from both brain and lymphoid tissue.

Materials and methods

Cells and plasmids

Preparation of glial cell cultures from human fetal brain has been described in detail (Major and Vacante, 1989). Briefly, primary (8–16 weeks gestation) human fetal glial cells (HFG) were grown in Eagle's minimum essential medium with 10% fetal bovine serum, L-glutamine (3.0 mg/ml), and antibiotics. Cells were grown at 37°C with 5% CO_2 in poly-D-lysine coated plasticware. Cultures in which greater than 98% of the cells stained with the astrocyte specific marker GFAP were used in these experiments. The Namalwa EBV positive human B cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). A3.01 human T cells were kindly provided by Malcolm Martin (NIH, Bethesda, MD). Both the Namalwa and

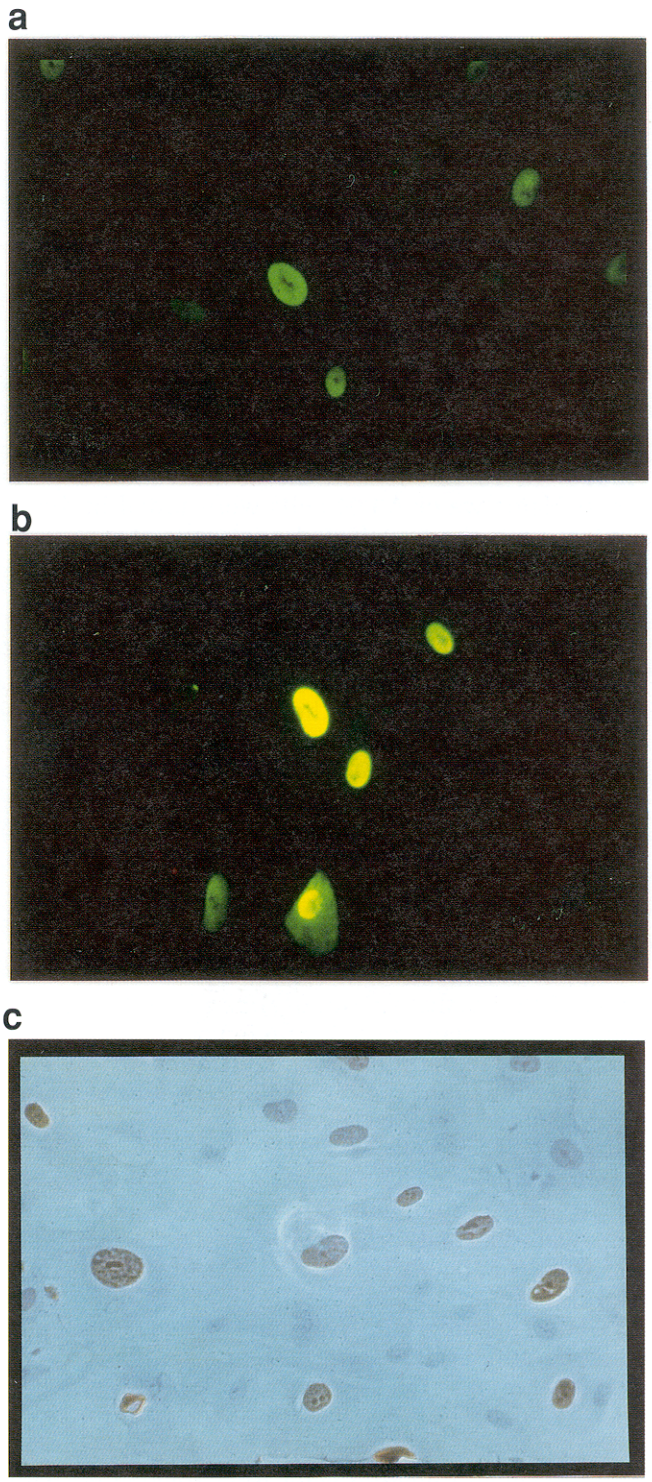

Figure 6 Immunofluorescence and *in situ* hybridization analyses of T, V, and DNA positive cells. (a) Representative field of T antigen positive cells. (b) Representative field of V antigen positive cells. (c) Representative field of *in situ* hybridization positive cells.

A3.01 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine (3.0 mg ml^{-1}), and antibiotics. The plasmid pM1CAT contains the regulatory region of the Mad-1 strain of JCV (Hind III to Pvu II) cloned into pA10CAT upstream of the reporter gene chloramphenicol acetyl transferase. Transcription from the JCV promoter is in the early orientation. pA10CAT has been described elsewhere (Gendelman *et al*, 1986).

Transfection and CAT assays
Cells were transfected in triplicate by calcium phosphate precipitation with 10 μg of pM1CAT DNA, 10 μg of a negative control construct (pA10CAT), or with 10 μg of calf thymus DNA. Following transfection the cells were grown for 48 h in media alone or in media containing TNF-α (1000 U ml^{-1}), IL-1β (10 U/ml), IL-2 (50 u ml^{-1}), GM-CSF (400 U ml^{-1}), IL-6 (500 u ml^{-1}), or PMA and the calcium ionophore A23187 at 10^{-6}M each. In the cocultivation experiments Namalwa or A3.01 cells were added in direct contact with the transfected glial cells at a ratio of 10:1 (lymphocytes:glial cells). The transfected cells were then washed 2× in phosphate buffered saline, pH 7.2 (PBS), 1× in mM Tris-HCL (pH 7.1), and lysed by successive freeze thaw cycles in 100 μl of 250 mM Tris-HCL (pH 7.1). After removal of cell debris by centrifugation the protein concentration in the cell extract was determined by the method of Bradford (Bradford, 1976). Equivalent amounts of protein were assayed for CAT enzyme activity using the fluor diffusion method of Neuman (Neuman *et al*, 1987). Briefly, 80μg of protein, diluted in 250 mM Tris-HCL (pH 7.1), was added to a reaction mixture containing 0.25 mM chloramphenicol, and 0.5 μCi (2.5 nM) of ^{3}H-acetyl coenzyme A. The mix was then overlayed with 5.0 ml liquid scintillation cocktail (Econofluor-2 Dupont) and counted in a β counter (Beckman) for 3 h. Efficiency of transfection within each experiment was controlled for by slot blot hybridization of DNA isolated from the transfected cells.

Infection
1×10^{4} cells growing on poly-D lysine coated coverslips were washed 2× in media containing 2% FCS and then incubated with 400 HAU of JCV (MAD-4 strain) in 1.0 ml media containing 2% FCS for 1.5 h at 37°C. The inoculum was removed and the cells were maintained in media with and without TNF-α at 1000 U ml^{-1}.

Immunofluorescent analysis of T and V antigens
At 5 days post infection cells were washed 3× with PBS and fixed for 10 min in ice cold acetone. Duplicate coverslips were then incubated with anti-SV40 T antigen antibody (PAB 416, Oncogene Sciences, 1:10), anti-SV antigen antibody (PAB 597, a generous gift of L Norkin, 1:100), or with negative control antibodies for 45 min at 37°C. The cross reactivity of these anti-SV40 antibodies with JCV proteins has been previously published (Major *et al*, 1987). The coverslips were then washed 3× in PBS and incubated with secondary goat anti-mouse antibody conjugated to FITC (Jackson Immunoresearch, West Grove, Pennsylvania, 1:15) for an additional 0.5 h. The coverslips were washed 3× in PBS and mounted on glass slides with 90% glycerol. T and V antigen positive cells were visualized on a Zeiss epifluorescent microscope.

In situ *hybridization*
In situ hybridization was performed as previously described (Aksamit *et al*, 1985). Briefly, cells grown on coverslips were washed in PBS and fixed for 0.5 h in 4% paraformaldehyde at room temperature. The cells were then dehydrated in serial ethanol washes. Cells were acid hydrolyzed in HCL, washed in Triton-X, subjected to limited protein digestion with pronase, and washed in glycine buffer. The cells were hybridized with 25–40 μl of a probe mixture that contained 50% formamide, 10% dextran sulfate, 0.4 mg ml^{-1} calf thymus DNA, 2 μg ml^{-1} biotinylated JCV DNA probe (ENZO Biochem), and 2× SSC (300mM sodium chloride and 30 mM sodium citrate). Probe DNA and cellular DNA were denatured by incubation at 85°C for 10 min. Hybridization was performed at 37°C overnight. Cells were then washed with 2× SSC for 2 min, with 0.1% Triton-X in PBS for 2 min, and in PBS for 3 min. Detection of the biotinylated probe was carried out immediately by direct affinity cytochemistry using the streptavidin-biotin-horseradish peroxidase kit (Detek I-hrp, ENZO, Biochem.). A fresh solution of diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen. Cells were then washed in PBS, counterstained with hematoxylin, dehydrated, and mounted.

Preparation of nuclear extracts
Cultures of human fetal glial cells ($1–10 \times 10^{8}$ cells) were grown for 48 h in the presence or absence of TNF-α (1000 U ml^{-1}). Nuclear extracts were prepared by a modification of the procedure of Dignam and has been previously described (Amemiya *et al*, 1989; Atwood *et al*, 1992; Dignam *et al*, 1983). The protein concentration of the samples was determined by the method of Bradford (Bradford, 1976).

Oligonucleotide probes and gel shift assays
The following oligonucleotide probes were synthesized on an Applied Biosystems (Foster City, California) 380A DNA synthesizer:

JCV NFκB:
(5′-GATCTGGAGGCCAGGGAAATTCCCTTGTTT-TA-3′)
mutant JC κB:
(5′-GATCTGGAGGCCAGGTCAATTAACTTGTTT-

TA-3′)
HIV-1 NFκB:
(5′-GGGACTTTCC-3′)
NF1/AP1:
5′-GGGATGGCTGCCAGCCAAGCATGAGCTCAT-ACC-3′

Double stranded oligonucleotides were labeled with γ-^{32}P-ATP and gel purified. The labeled JC NFκB probe (40,000 CPM) was incubated with 20 μg of nuclear extract from untreated or TNF-α treated cells in the presence or absence of a 10-fold molar excess of unlabeled homologous probe, unlabeled mutant probe, or unlabeled HIV-1 NFκB probe. The DNA binding reactions also contained 10 mM Tris-HCL (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM DTT, 10% glycerol (v/v), and 4 μg of the non-specific competitor, poly (dI-dC) (Pharmacia). The reactions were carried out at room temperature for 15 min and electrophoresed on a 6% polyacrylamide Tris-glycine gel. The gel was dried and samples visualized by autoradiography with Kodak ×AR-5 film with an intensifying screen.

The NF1/AP1 probe was used in a similar manner except that only 10 μg nuclear extracts were used in the reactions.

Supershift assays

The radiolabeled JC NFκB probe was incubated for 15 min at room temperature with 20 μg of nuclear extract from untreated or TNF-α treated HFG cells under the conditions described above. After the complexes were formed, 1.0 μl of either rabbit anti-p50 serum (a generous gift from Ulrich Siebenlist), rabbit anti-p65 serum (Santa Cruz Biochem Inc, Santa Cruz, California), or rabbit hyperimmune anti-galactocerebroside serum, as a negative control, were added to the reactions and incubated on ice for an additional 30 min. The complexes were resolved on 6% Tris-glycine gels as described above.

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References

Ahmed S, Chowdhury M, Khalili K (1990a). Regulation of a human neurotropic virus promoter, JCVE: identification of a novel activator domain located upstream from the 98 bp enhancer promoter region. *Nucleic Acids Res* **18**: 7417–7423.

Ahmed S, Rappaport J, Tada H, Kerr D, Khalili K (1990b). A nuclear protein derived from brain cells stimulates transcription of the human neurotropic virus promoter, JCVE, *in vitro*. *J Biol Chem* **265**: 13899–13905.

Aksamit AJ, Mourrain P, Sever JL, Major EO (1985). Progressive multifocal leukoencephalopathy: investigation of three cases using *in situ* hybridization with JC virus biotinylated DNA probe. *Ann Neurol.* **18**: 490–496.

Amemiya K, Traub R, Durham L, Major EO (1989). Interaction of a nuclear factor-1-like protein with the regulatory region of the human polyomavirus JC virus. *J Biol Chem.* **264**: 7025–7032.

Amemiya K, Traub R, Durham L, Major EO (1992). Adjacent nuclear factor-1 and activator protein binding sites in the enhancer of the neurotropic JC virus. A common characteristic of many brain-specific genes. *J Biol Chem* **267**: 14204–14211.

Atwood WJ, Tornatore C, Traub R, Conant K, Major E (1994). Stimulation of HIV-1 gene expression and induction of NF-kB (p50/p65) in TNF-alpha treated human fetal glial cells. *AIDS Research and Human Retroviruses* **10**: 1207–1211.

Atwood WJ, Amemiya K, Traub R, Harms J, Major EO (1992). Interaction of the human polyomavirus, JCV, with human B-lymphocytes. *Virology* **190**: 716–723.

Baeuerle PA (1991). The inducible transcription activator NF-kB:regulation by distinct protein subunits. *Biochim Biophys Acta* **1072**: 63–80.

Berger JR, Kaszovitz B, Post MJ, Dickinson G (1987). Progressive multifocal leukoencephalopathy associated with human immunodeficiency virus infection: A review of the literature with a report of sixteen cases. *Ann Intern Med* **107**: 78–87.

Bours V, Burd PR, Brown K, Villalobos J, Park S, Ryseck RP, Bravo R, Kelly K, Siebenlist U (1992a). A novel mitogen-inducible gene product related to p50/p105-NF-kappa B participates in transactivation through a kappa B site. *Mol Cell Biol* **12**: 685–695.

Bours V, Franzoso G, Brown K, Park S, Azarenko V, Tomita-Yamaguchi M, Kelly K, Siebenlist U (1992b). Lymphocyte activation and the family of NF-kB transcription factor complexes. *Curr Top Microbiol Immunol* **182**: 411–420.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Brown K, Park S, Kanno T, Franzoso G, Siebenlist U (1993). Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc Natl Acad Sci USA* **90**: 2532–2536.

Chowdhury M, Taylor JP, Tada H, Rappaport J, Wong-Staal F, Amini S, Khalili K (1990). Regulation of the human neurotropic virus promoter by JCV-T antigen and HIV-1 tat protein. *Oncogene* **5**: 1737–1742.

Conant K, Atwood WJ, Traub R, Tornatore CS, Major EO

(1994). Expression of HIV-1 in human fetal astrocytes: Role of PKC and NFkB. *Virology.* **205**: 586–590.

Dignam JD, Lebovitz RM, Roeder RG (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**: 1475–1489.

Dorries K, Vogel E, Gunther S, Czub S (1994). Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals. *Virology* **198**: 59–70.

Feigenbaum L, Khalili K, Major E, Khoury G (1987). Regulation of the host range of human papovavirus JCV. *Proc Natl Acad Sci USA* **84**: 3695–3698.

Ganchi PA, Sun S, Green WC, Ballard (1993). A novel NF-kB complex containing p65 homodimers: Implications for transcriptional control at the level of subunit dimerization. *Mol Cell Biol* **13**: 7826–7835.

Gendelman HE, Phelps W, Feigenbaum L, Ostrove JM, Adachi A, Howley PM, Khoury G, Ginsberg Harold S, Martin Malcolm A (1986). Trans-activation of the human immunodeficiency virus long terminal repeat DNA viruses. *Proc Natl Acad Sci USA* **83**: 9759–9763.

Henson JW (1994). Regulation of the glial-specific JC virus early promoter by the transcription factor Sp 1. *J Biol Chem* **269**: 1046–1050.

Houff SA, Major EO, Katz DA, Kufta CV, Sever JL, Pittaluga S, Roberts JR, Gitt J, Saini N, Lux W (1988). Involvement of JC virus-infected mononuclear cells from the bone marrow and spleen in the pathogenesis of progressive multifocal leukoencephalopathy. *N Engl J Med* **318**: 301–305.

Kawakami K, Scheidereit C, Roeder RG (1988). Identification and purification of a human immunoglobulin enhancer binding protein (NFkB) that activates transcription from a human immunodeficiency virus type 1 promoter *in vitro*. *PNAS* **85**: 4700–4704.

Kumar G., Srivastava DK, Tefera W (1994). A 70-KDA to 80-KDA glial-cell protein interacts with the AGGGAAGGGA domain of the JC virus early promoter only in the presence of the neighboring cis DNA elements. *Virology* **203**: 116–124.

Kure K, Llena JF, Lyman WD, Soeiro R, Weidenheim KM, Hirano A, Dickson DW (1991). Human immunodeficiency virus-1 infection of the nervous system: an autopsy study of 268 adult, pediatric, and fetal brains. *Hum Pathol* **22**: 700–710.

Lenardo M, Pierce JW, Baltimore D (1987). Protein binding sites in Ig enhancers determine transcriptional activity and inducibility. *Science* **236**: 1573–1577.

Lenardo MJ, Baltimore D (1989). Nf-kB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**: 227–229.

Lenardo MJ, Fan C, Maniatis T, Baltimore D (1989). The involvement of NF-kB in B-interferon gene regulation reveals its role as a widely inducible mediator of signal transduction. *Cell* **57**: 287–294.

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.

Major EO, Vacante DA, Traub RG, London WT, Sever JL (1987). Owl monkey astrocytoma cells in culture spontaneously produce infectious JC virus which demonstrates altered biological properties. *J Virol* **61**: 1435–1441.

Major EO, Amemiya K, Elder G, Houff SA (1990). Glial cells of the human developing brain and B cells of the immune system share a common DNA binding factor for recognition of the regulatory sequences of the human polyomavirus, JCV. *J Neurosci Res* **27**: 461–471.

Major EO, Amemiya K, Tornatore CS, Houff SA, Berger JR (1992). Pathogenesis and molecular biology of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain. *Clin Microbiol Rev* **5**: 49–73.

Mazlo M, Tariska I (1980). Morphological demonstration of the first phase of polyomavirus replication in oligodendroglia cells of human brain in progressive multifocal leukoencephalopathy (PML). *Neuropathology* **49**: 133–143.

Mercurio F, DiDonato J, Rosette C, Karin M (1993). p105 and p98 precursor proteins play an active role in NF-kB-mediated signal transduction. *Genes Dev* **7**: 705–718.

Molitor J, Walker W, Doerre S, Ballard D, Greene W (1990). NF-kB: A family of inducible and differentially expressed enhancer-binding proteins in human T cells. *Proc Natl Acad Sci USA* **87**: 10028–10032.

Neuman J, Morency C, Russian K (1987). A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* **5**: 444–447.

Padgett B, ZuRhein G, Walker D, Echroade R, Dessel B (1971). Cultivation of papova-like virus from human brain with progressive multifocal leukoencephalopathy. *Lancet* **I**: 1257–1260.

Ranganathan PN, Khalili K (1993). The transcriptional enhancer element, kappa B, regulates promoter activity of the human neurotropic virus, JCV, in cells derived from the CNS. *Nucleic Acids Res* **21**: 1959–1964.

Renner K, Leger H, Wegner M (1994). The POU domain protein TST-1 and papovaviral large tumor antigen function synergistically to stimulate glia-specific gene expression of JC virus. *Proc Natl Acad Sci USA* **91**: 6433–6437.

Richardson EP, Webster H de F (1983). Progressive multifocal leukoencephalopathy: its pathological features. In: Sever JL, Madden G (eds). *Polyomaviruses and Human Neurological Disease.* Alan R Liss Inc: New York, pp 191–203.

Schneider EM, Dorries K (1993). High frequency of polyomavirus infection in lymphoid cell preparations after allogeneic bone marrow transplantation. *Transplant Proc* **25**: 1271–1273.

Sock E, Wegner M, Grummt F (1991). DNA replication of human polyomavirus JC is stimulated by NF-1 *in vivo*. *Virology* **182**: 298–308.

Tada H, Khalili K (1992). A novel sequence-specific DNA-binding protein, LCP-1, interacts with single-stranded DNA and differentially regulates early gene expression of the human neurotropic JC virus. *J Virol* **66**: 6885–6892.

Tamura T, Inoue T, Nagata K, Mikoshiba K (1988). Enhancer of human polyoma JC virus contains nuclear factor-1 binding. *Biochem Biophys Res Commun* **157**: 419–425.

Tamura T, Aoyama A, Inoue T, Miura M, Kikoshiba K (1990). A new transcription element in the JC virus enhancer. *J Gen Virol* **71**: 1829–1833.

Tornatore C, Berger JR, Houff SA, Curfman B, Meyers K, Winfield D, Major EO (1992). Detection of JC virus DNA in peripheral lymphocytes from patients with and

without progressive multifocal leukoencephalopathy. *Ann Neurol* **31**: 454–462.

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.

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

Vitkovic L, Wood GP, Major EO, Fauci AS (1991). Human astrocytes stimulates HIV-1 expression in a chronically infected promonocyte clone via Il-6. *AIDS Res Hum Retroviruses* **7**: 723–727.

Walker DL, Padgett BL (1983). The epidemiology of human polyomaviruses. In: Sever JL, Madden D (eds). *Polyomaviruses and Human Neurological Disease.* Alan R Liss Inc: New York, pp 99–106.

Walker DL, Frisque RJ (1986). The biology and molecular biology of JC virus. In: Salzman NP (ed). *The Papovaviridae.* Plenum Press: London and New York, pp 327–377.

Wegner M, Drolet DW, Rosenfeld MG (1993). Regulation of JC virus by the POU-domain transcription factor Tst-1: Implications for progressive multifocal leukoencephalopathy. *Proc Natl Acad Sci USA* **90**: 4743–4747.

Weiner LP, Narayan O, Penney JB Jr, Walker DL, Dupont B (1973). Papovavirus of JC type in progressive multifocal leukoencephalopathy. *Arch Neurol* **29**: 1–3.

Wesselingh SL, Power C, Glass JD, Tyor WR, McArthur JC, Farber JM, Griffin JW, Griffin DE (1993). Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. *Ann Neurol* **33**: 576–582.