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JC Virus binds to primary human glial cells, tonsillar stromal cells, and B-lymphocytes, but not to T lymphocytes

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The human polyomavirus, JCV, is the etiological agent of the fatal central nervous system demyelinating disease, progressive multifocal leukoencephalopathy (PML). In PML patients, JC Virus (JCV) can be detected in glial cells in the central nervous system (CNS); in B-lymphocytes in the peripheral blood, bone marrow, spleen, and tonsil; and in tonsillar stromal cells. *In vitro*, JCV infects glial cells, tonsillar stromal cells, and to a limited extent B-lymphocytes. The presence or absence of as yet unidentified cell type specific transcription factors contributes to the restricted tropism of JCV for these cell types. However, several studies indicate that cell surface receptors may also contribute to the limited host range of JCV. To examine this latter possibility we measured the binding of purified JCV virions to primary cultures of glial cells, tonsillar stromal cells, peripheral blood lymphocytes, and to several established cell lines. Our results demonstrate that JCV binds to primary glial cells, stromal cells, and B cells, but does not bind to primary T cells. In contrast, JCV bound to all cell lines tested, including the Namalwa B cell line and the Jurkat T cell line. These data are novel and demonstrate that JCV selectively interacts with cells *in vivo* that are known to be susceptible to infection. This selectivity appears to be lost when one examines virus binding to a variety of human, monkey, or mouse tumor cell lines. We next examined the susceptibility of primary peripheral blood lymphocytes and the Namalwa B cell line to infection with JCV. Our results demonstrate that the majority of infectious JCV virions remain cell surface associated and do not efficiently establish infection of B cells. This may explain the *in vivo* observation that JCV DNA is frequently detected in association with lymphocytes by PCR but that JCV mRNA is rarely detected in association with lymphocytes by reverse transcriptase PCR. These results also confirm previous data regarding the association of JCV with human B cells *in vivo* and support the hypothesis that B cells may be involved in trafficking of JCV to the CNS. *Journal of NeuroVirology* (2000) **6,** 127–136.

Keywords: JC virus; B lymphocytes; virus receptors; progressive multifocal leukoencephalopathy

Introduction

Seroepidemiological studies indicate that 70% of the human population worldwide is infected with the human polyomavirus, JCV (Major *et al*, 1992; Tornatore *et al*, 1994; Walker and Frisque, 1986). The mode of virus transmission is unknown, and no clinical illness has been associated with primary infection. Like other polyomaviruses, infection with JCV is associated with the establishment of lifelong latent or persistent infections (Norkin, 1982; Walker and Frisque, 1986). Reactivation of JCV in chronically immunosuppressed patients leads to virus dissemination to the central nervous system and subsequent infection of glial cells. The primary targets of virus infection in the CNS are oligodendrocytes and astrocytes. The fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML), results from lytic destruction of oligodendrocytes by JCV. JCV has also been associated with several neural and non-neural tumors, including medulloblastoma, neuroblastoma, glioblastoma, oligoastrocytoma, pineocytoma, and B cell lymphoma (Major *et al*, 1992; Rencic *et al*, 1996; Tornatore *et al*, 1994). Recently, JCV DNA sequences corre-

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sponding to the T antigen coding region were detected in the mucosa of human colon and in a significant proportion of cells derived from colorectal cancers (Laghi *et al*, 1999).

The site of virus latency, and the mechanisms by which JCV is reactivated, gains access to the CNS, and infects glial cells are not known. Excretion of JCV in the urine of immunosuppressed individuals is common and hence the urogenital system is suspected as a potential site of virus latency or persistence (Markowitz *et al*, 1991, 1993). A specific cell type harboring virus in the urogenital system has not been described. The distribution of demyelinated lesions in the CNS of PML patients suggests that virus spreads to the CNS hematogenously (Astrom *et al*, 1958). This is supported by several studies showing virus in the peripheral blood of the majority of PML patients and in increased frequency in AIDS patients (Dubois *et al*, 1996a, b; Ferrante *et al*, 1996; Monaco *et al*, 1996; Pietzuch *et al*, 1996; Tornatore *et al*, 1992). In the peripheral blood JCV is exclusively associated with B cells (Monaco *et al*, 1996). In addition, JCV infected B cells have been detected by *in situ* hybridization in bone marrow, spleen, and brain of patients with PML (Houff *et al*, 1988). These observations have led to the suggestion that virus may be latent in lymphoid organs and that B-lymphocytes may be a vehicle for the dissemination of virus to the CNS. Others have observed JCV DNA in normal brain and have suggested that the CNS may be another site of virus latency (Elsner and Dorries, 1992; Mori *et al*, 1991; Quinlivan *et al*, 1992; Vago *et al*, 1996; White *et al*, 1992).

In vivo, JCV infection is restricted to oligodendrocytes, astrocytes, and B-lymphocytes (Major *et al*, 1992; Monaco *et al*, 1996). This highly restricted cell type specificity is also seen *in vitro*, as virus infection is restricted to primary cultures of human glial cells, and to a few established human glial and B cell lines (Atwood *et al*, 1992; Major *et al*, 1992; Monaco *et al*, 1996). Although it has been suggested that cell type specific transcription factors play a role in determining JCV tropism for glial cells and B cells, no such factor has been convincingly demonstrated. A role for specific cellular receptors in determining JCV tropism is suggested by several studies demonstrating a block to infection of non-permissive cells at the level of virus binding and entry. These studies found that some human and primate cell lines that were resistant to infection with JCV could replicate JCV DNA it if was artificially introduced into the cell either by microinjection or transfection (Feigenbaum *et al*, 1987; Itoh, 1995).

Our laboratory has characterized the cellular receptor for JCV on human glial cells as an N-linked glycoprotein containing terminal α (2–6) linked sialic acids (Liu *et al*, 1998a, b). To investigate the role of receptors in determining JCV specific cellular tropism we measured the binding of JCV to primary human fetal glial cells, primary human tonsillar stromal cells, primary human B cells, primary human T cells, and to a wide variety of cell lines. Our results demonstrate that JCV selectively binds to primary human fetal glial cells, primary tonsillar stromal cells, and to primary human B cells, but does not bind to primary human T cells. In contrast, virus bound to all cell lines tested, including the Namalwa B cell line, and the Jurkat T cell line. We next tested the susceptibility of B cells to infection with JCV using an indirect infectivity assay and RT–PCR. We demonstrate that the majority of JCV virions remain cell surface associated and that only a minority of virus establishes infection.

Results

Virus binding to primary cells

Previous studies using *in situ* hybridization, immunocytochemistry, or PCR have localized JC Virus DNA and antigen to glial cells, B lymphocytes, and tonsillar stromal cells. In addition, JCV has been shown to replicate in cultures of primary human fetal glial cells, tonsillar stromal cells, and to a limited extent, in human B cells. The tropism of JCV for these cell types may be due to the presence or absence of specific transcription factors, the presence or absence of a specific cellular receptor, or both. To examine the latter possibility we measured the binding of FITC-labeled JCV to primary cultures of human fetal glial cells, tonsillar stromal cells, and to B cells and T cells isolated from peripheral blood by flow cytometry. As expected, JCV bound to both primary human fetal glial cells and to tonsillar stromal cells (Figure 1). We next used triple color flow cytometry to measure FITC-labeled JCV binding to B cells and T cells isolated from peripheral blood. An analysis of the peripheral blood lymphocytes using phycoerythrin labeled CD19 as a B cell specific marker and Texas red labeled CD3 as a T cell specific marker indicated that the PBLs contained 10.8% B cells and 83% T cells (Figure 2, upper left panel). All of the FITC-labeled JCV binding co-localized with CD19 positive B cells (Figure 2, bottom panel). In contrast, all of the CD3 positive T cells were negative for virus binding (Figure 2, upper right panel). These experiments indicate that T cells lack specific binding sites for JCV and explain the strong association of JCV with glial cells, tonsillar stromal cells, and B cells *in vivo* and the lack of an association of JCV with T cells.

Virus binding to cell lines

We next asked whether the specificity of virus binding to distinct cell types *in vivo* correlated with binding of virus to different cell lines. Similar to the results with primary cells virus bound to both the SVG glial cell line and to the Namalwa B cell line (Figure 3). In contrast to the results with primary

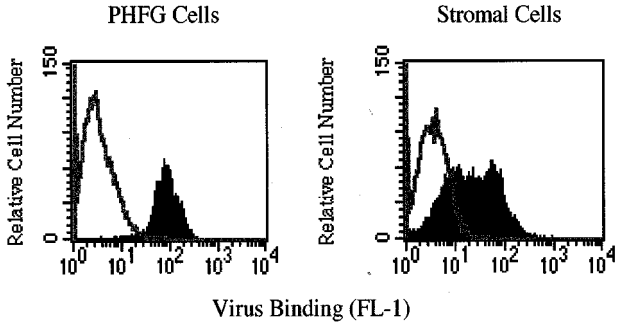

Figure 1 Virus binds to primary human fetal glial cells (PHGF) and to primary human tonsillar stromal cells. PHFG and tonsillar stromal cells were incubated on ice with equivalent amounts of FITC-labeled JCV (solid histograms in each panel) or with FITC-labeled BSA as a negative control (open histogram in each panel). Virus binding was analyzed by flow cytometry on a FACScalibur flow cytometer. Virus bound to both PHFG and to stromal cells. PHFG and stromal cells are known to be susceptible to infection with JCV. Relative cell number is plotted on the Y-axis and the mean fluorescence intensity of FITC-labeled virus binding is plotted on the X-axis (Fl-1).

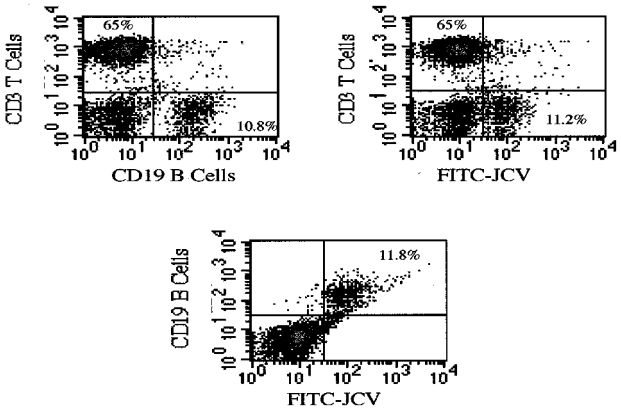

Figure 2 JCV binds to B cells and not to T cells isolated from peripheral blood. Peripheral blood mononuclear cells (PBMC) were isolated from a normal human volunteer by centrifugation over Ficoll-hypaque. The cells were incubated with a T cell specific monoclonal antibody conjugated to Quantum Red (anti-CD3), a B cell specific monoclonal antibody conjugated to PE (anti-CD19), and with FITC-labeled JCV. A density plot of CD3 *versus* CD19 demonstrated that 65% of the isolated PBMC were T cells and 11% were B cells (upper left panel). FITC-labeled JCV did not bind to CD3+ T cells (upper right panel). In contrast, all of the CD19 positive B cells were also positive for virus binding (bottom panel).

cells virus also bound to Jurkat T cells, HeLa cells, mouse 3T3 cells, and monkey CV1 cells (Figure 3). There were no significant differences in the amount of virus binding to any of these cell lines. The ability of virus to bind to this wide variety of cell types *in vitro* did not correlate with their known susceptibility to infection with JCV.

Infection of B cells by JCV

As JCV is associated with B cells and not T cells *in vivo* we examined the ability of JCV to infect

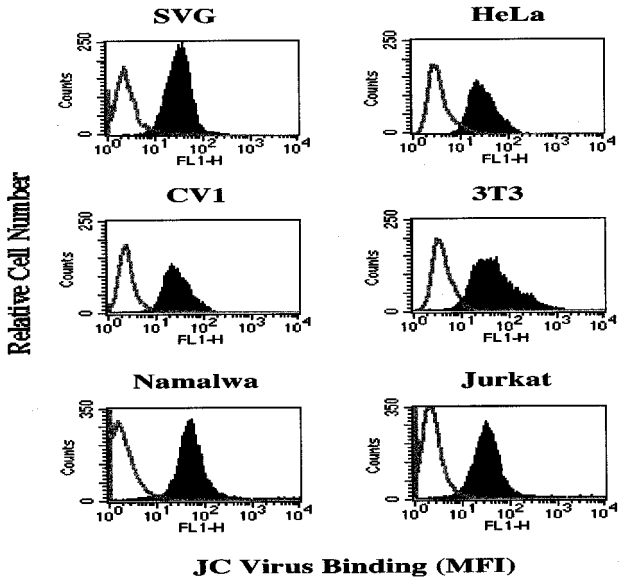

Figure 3 Virus binding to cell lines does not correlate with their susceptibility to infection. SVG cells (human glial), HeLa cells (human carcinoma), CV1 cells (monkey kidney), 3T3 cells (mouse kidney), Namalwa cells (human B cell), or Jurkat cells (human T cell) were incubated on ice with equivalent amounts of FITC-labeled JCV (solid histograms in each panel) or with FITC-labeled BSA as a negative control (open histogram in each panel). Virus binding was analyzed by flow cytometry on a FACScalibur flow cytometer. No significant differences in virus binding were detected on any of the cell lines tested regardless of their susceptibility to infection.

primary cultures of human peripheral blood lymphocytes and the Namalwa B cell line. Previous data indicated that infection of B cells by JCV was inefficient with only 1% or less of the cells becoming infected. Based on these data we decided to use an indirect infectivity assay and a sensitive RT–PCR assay to score virus infected cells. In the indirect assay, human PBMC or Namalwa B cells were infected with 2048 HAU/ml of JCV for 2 h at 37°C. The cells were then washed three times in PBS, and cultured for 24 h in growth media. At 24 h post-infection the cells were incubated in trypsin to inactivate any remaining cell surface associated virus. Untreated and trypsin treated PBMC and Namalwa B cells were then co-cultured with SVG cells for 7 days. The SVG cells were then plated onto coverslips and the ability of PBMC or Namalwa cells to transmit infection to the SVG cells was scored by indirect immunofluorescence analysis of V antigen expression. PBMC and Namalwa B cells readily transmitted infection to SVG cells (Figure 4). Treatment of the PBMCs or the Namwala B cells with trypsin at 24 h post-infection significantly reduced the ability of these cells to transmit JCV to SVG cells (Figure 4). This indicates that the majority of transmitted virus was cell surface associated.

We next examined the susceptibility of Namalwa B cells to infection with JCV by RT–PCR. Namalwa cells were incubated with JCV for 2 h at 37°C,

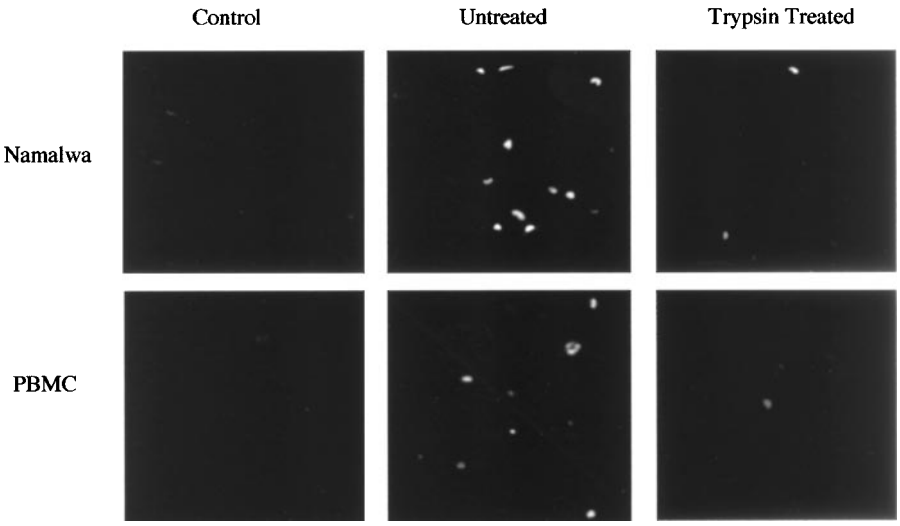

Figure 4 Namalwa B cells and PBMC transmit JCV infection to human glial cells. Namalwa B cells and PBMC were incubated with JCV for 2 h at 37°C, washed extensively and grown overnight. The cells were then harvested, washed in PBS, and then incubated in either PBS or in Trypsin for 10 min at 37°C. The cells were then washed several times in media containing 10% FCS and then co-cultured with SVG cells growing on coverslips. At 1 week post infection SVG cells were fixed and stained for the presence of JCV V antigen. Namalwa cells and PBMC readily transmitted infection to SVG cells. Trypsinization of either Namalwa B cells or PBMC 24 h post-infection significantly reduced the transmission of virus to SVG cells indicating that the majority of virus being transmitted is cell surface associated virus.

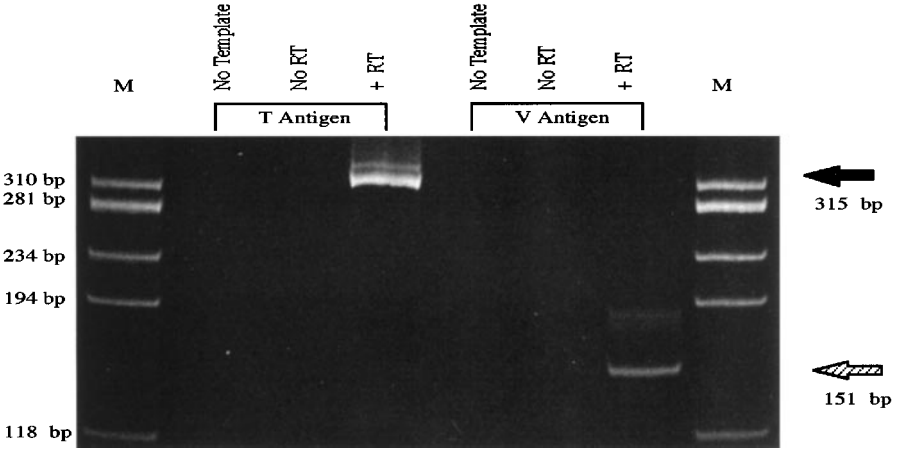

Figure 5 JCV T and V antigen mRNA are expressed in Namalwa B cells. Namalwa B cells were infected with JCV and total RNA harvested at 24 and 72 h post-infection. The RNA was reverse transcribed and the resulting cDNA amplified with primers to the JCV T antigen gene (315 bp product, indicated by a solid arrow) or the JCV V antigen gene (150 bp product, indicated by a hatched arrow). RT–PCR products were resolved on 5% polyacrylamide gels. M, molecular weight markers.

washed three times in PBS, and total RNA harvested at 24 and 72 h post-infection. The RNA was reverse transcribed using random primers and then subjected to nested PCR using oligonucleotide primers specific for either JCV T antigen or JCV V antigen cDNA. JCV specific cDNA PCR products corresponding to T antigen (315 bp product) and V antigen (151 bp) were detected by nested RT–PCR (Figure 5). We did not detect any PCR products following a single round of PCR amplification (not shown). This indicates that JCV replicates to low levels in human B cells (Figure 5). These data are consistent with other reports indicating that B cells are susceptible to low levels of JCV infection.

Neuraminidase inhibits the ability of B cells to transmit JCV infection to glial cells

An unidentified N-linked glycoprotein containing terminal α (2–6) linked sialic acids is a major component of the glial cell receptor for JCV.

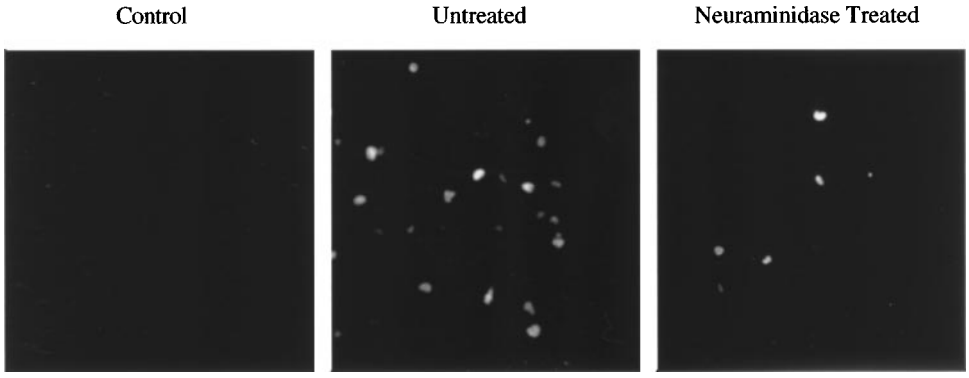

Figure 6 Neuraminidase inhibits the ability of B cells to transmit JCV infection to glial cells. Untreated and neuraminidase treated Namalwa B cells were infected for JCV. At 1 week post-infection the cells were lysed and the lysates used to infect the human glial cell line, SVG. At 3 weeks post-infection 26.7% of the SVG cells infected with lysates from untreated Namalwa B cells were V antigen positive (middle panel). In contrast, only 10.2% of the SVG cells infected with lysates from neuraminidase treated Namalwa B cells were V antigen positive (right panel). No SVG cells became infected when lysates of uninfected Namalwa B cells were used as the innoculum (left panel).

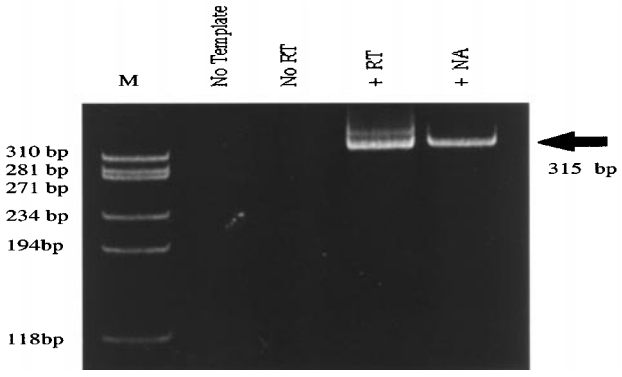

Figure 7 Neuraminidase does not inhibit infection of B cells by JCV. Namalwa B cells were either untreated or treated with neuraminidase. The cells were then infected with JCV and at 24 h post-infection total RNA was extracted from the cells. The RNA was reverse transcribed and the resulting cDNA amplified with T antigen specific primers. The expected 315 bp T antigen specific cDNA product was detected in Namalwa B cells (arrow, as indicated). Neuraminidase treatment of the cells did not significantly reduce our ability to amplify T antigen specific cDNA by this very sensitive nested RT–PCR assay (as indicated).

Infection of glial cells by JCV is completely abrogated by pre-treatment of the glial cells with neuraminidase. To determine the effect of neuraminidase on virus infectivity of Namalwa cells we treated the cells for 1 h with crude neuraminidase. Untreated and neuraminidase treated cells were then infected with 2046 HAU/ml of JCV for 1 h at 4°C. At 1 week post-infection the cells were harvested and lysed in deoxycholic acid and the lysates used to infect SVG cells. We began to see V antigen expression in the SVG cells at 2 weeks post-infection using lysates from untreated Namalwa cells. In contrast, no V antigen expression in the SVG cells was detected at this time point using lysates from neuraminidase treated Namalwa cells. By 3 weeks post-infection 26.7% of the SVG cells infected with lysates from untreated Namalwa cells expressed V antigen and 10.2% of the SVG cells infected with lysates from neuraminidase treated Namalwa cells expressed V antigen (Figure 6).

To ascertain whether these results were due to a reduction in infectivity or to a reduction in cell surface associated virus we infected untreated and neuraminidase treated Namalwa cells with JCV and at 24 h post-infection harvested total RNA. Identical amounts of RNA from untreated and treated cells was reverse transcribed and the resulting cDNA amplified with JCV T antigen specific primers. Neuraminidase treatment of the Namalwa cells did not significantly reduce the amount of JCV T antigen specific RT–PCR product (Figure 7).

Discussion

Hematogenous spread of JCV to the CNS was first suggested in 1958 by Astrom and Richardson (Astrom *et al*, 1958). Thirty years later Houff and Major identified JCV nucleic acids and protein in B lymphocytes in the bone marrow, spleen, and brain of a PML patient (Houff *et al*, 1988). This led to the hypothesis that B lymphocytes were a potential reservoir of JCV outside of the CNS and that they may be responsible for trafficking JCV to the CNS (Houff *et al*, 1988). In support of this hypothesis, several groups have demonstrated a high degree of association between JCV and B cells in the peripheral blood of immunosuppressed patients (Dubois *et al*, 1996a, b; 1997, 1998; Monaco *et al*, 1996; Pietzuch *et al*, 1996; Tornatore *et al*, 1992). It has also been shown that primary human B cells and B cell lines can support a low level of JCV

multiplication (Atwood *et al*, 1992; Monaco *et al*, 1996). Recently, JCV DNA was detected in a significant proportion of tonsillar tissue (stromal tissue and B-lymphocytes) from normal patients undergoing routine tonsillectomy and stromal cells isolated from these tonsils have been shown to support JCV multiplication (Monaco *et al*, 1996, 1998).

Our laboratory has focused on understanding the early events of JCV infection including the interactions of JCV with specific cellular receptors. We have determined that JCV binds specifically to an N-linked glycoprotein containing terminal α (2–6) linked sialic acids on human glial cells (Liu *et al*, 1998). The identity of this N-linked glycoprotein is currently under investigation. To begin to assess the role of virus receptors in determining cellular tropism we measured the binding of labeled JCV to primary human glial cells, primary human tonsillar stromal cells, primary human B cells, and primary human T cells. Virus bound to glial cells, stromal cells, and B cells, but did not bind to T cells. Interestingly all of the CD19 positive B cells bound virus. This indicates that virus is interacting with a ubiquitous B cell surface receptor rather than interacting only with B cells carrying JCV specific immunoglobulins. Note that the sero-positivity of the donor was not determined. The inability of virus to bind to T cells suggests either that T cells lack a specific virus receptor or that T cells express receptors that are inhibitory to virus binding. In either case, the specificity of virus binding to the primary B cells and not to T cells is consistent with the known association of virus for B cells and not T cells *in vivo* (Monaco *et al*, 1996).

We next analyzed virus binding to several cell lines, including SVG cells, HeLa cells, CV1 cells, mouse 3T3 cells, Namalwa B cells, and Jurkat T cells. In contrast to the results using primary B and T cells, virus bound to both the Namalwa B cell line and to the Jurkat T cell line. Virus also bound to SVG cells, HeLa cells, monkey CV1 cells, and mouse 3T3 cells. These data indicate that the specificity of virus binding seen *in vivo* is not maintained on cell lines. This result was not wholly unexpected as tumor cells and tumor cell lines often express increased levels of a number of cell surface molecules, including sialic acids.

We next explored the possibility that JVC receptors on B cells and glial cells were conserved. Previous data has demonstrated that infection of B cells by JCV is very inefficient with less than 1% of the cells actually becoming infected (Monaco *et al*, 1996). To study the effects of enzymes such as neuraminidase on inhibiting infection, we used an indirect virus infectivity assay and RT–PCR. In the indirect assay we first infected PBMC and Namalwa B cells with JCV, washed the cells extensively, and at 24 h post-infection, passaged the infected cells to cultures of highly permissive glial cells. At several time points post-co-culture the glial cells were plated onto coverslips and assayed for expression of JCV V antigen by indirect immunofluorescence assay. At 7 days post-co-culture a significant proportion of glial cells expressed JCV V antigen indicating that the PBMC and the Namalwa B cells both readily transmitted JCV to the highly susceptible glial cells. To determine whether the transmitted virus was derived from virus that had replicated in the B cells or from contaminating input virions, duplicate PBMC and Namalwa B cell cultures were infected with virus, washed extensively, and at 24 h post-infection, incubated for 10 min in trypsin to inactivate any remaining cell surface associated virus. Note that trypsin does not cleave specific JCV receptors as pre-treatment of cells with trypsin does not inhibit infection. Treatment of the PBMC and the Namalwa B cells with trypsin at 24 h post-infection almost completely abrogated the ability of the B cells to transmit infection to glial cells. This indicates that the majority of virions remained cell surface associated for at least 24 h post-infection.

We next used RT–PCR to study the susceptibility of Namalwa B cells to infection with JCV. The cells were infected and total RNA harvested at 24 and 72 h post-infection. The RNA was reverse transcribed and the resulting cDNA amplified with primers specific for either T antigen cDNA or V antigen cDNA. After a single round of RT–PCR no JCV T or V antigen cDNA was detected. We then re-amplified the first round of RT–PCR products with nested T and V antigen primers. At 24 h post-infection a predicted 315 bp T antigen specific cDNA was amplified and at 72 h post-infection the predicted 151 bp V antigen specific cDNA was amplified by nested PCR. These data confirm previous reports demonstrating that JCV establishes a low level productive infection in human B cells (Monaco *et al*, 1996). The RT–PCR products detected did not result from amplification of contaminating genomes as controls without the addition of reverse transcriptase were performed. Furthermore, the T antigen primers used in both the first and second rounds of amplification span an intron and therefore eliminate the possibility of amplifying contaminating DNA.

As neuraminidase treatment of glial cells completely abrogates infection by JCV we tested the effects of this enzyme at inhibiting infection of Namalwa B cells (Liu *et al*, 1998). In our first experiment we treated Namalwa cells with neuraminidase and then infected them with JCV. At 1 week post-infection cell lysates were prepared and the lysates used to infect highly susceptible glial cells. At 2 weeks post-infection we detected V antigen staining in the SVG cells infected with lysates from untreated Namalwa cells but not in SVG cells infected with lysates from neuraminidase treated Namalwa cells. By 3 weeks post-infection V

antigen expression was reduced twofold in SVG cells infected with lysates from neuraminidase treated Namalwa cells when compared to control infected cells. As JCV is very highly cell associated the effect of neuraminidase could be due to a reduction in cell associated virus and not to a reduction in infectivity. To directly address this we used a qualitative RT–PCR assay to distinguish between these possibilities. Neuraminidase treatment of Namalwa cells did not significantly reduce our ability to amplify T antigen cDNA at 24 h post-infection. This suggests that the major effect of neuraminidase at reducing the transmission of JCV from B cells to glial cells was at the level of the cell surface. This result, together with our data demonstrating that JCV remains sensitive to inactivation by trypsin for at least 24 h post-infection, suggests that a significant proportion of B cell associated virus may be cell surface associated and that only a minority of the virus actually establishes infection. We have studied the kinetics of JCV internalization into highly permissive glial cells and our results demonstrate that JCV enters into a trypsin insensitive compartment by 10 min post-adsorbtion and completely internalizes within 1 h (our unpublished data). When taken together our results suggest that the interactions between JCV and B cells may be fundamentally different and inherently less efficient than the interaction between JCV and highly permissive glial cells. Our results may also explain why JCV DNA but not mRNA is frequently detected in association with lymphocytes *in vivo*. The identification of a specific glial cell receptor for this important human pathogen should clarify the role of cellular receptors in determining the tropism of JCV for diverse cell types and human tissues.

Materials and methods

Cells, virus, and antibody

Primary cultures of human fetal glial cells (PHFG) were a kind gift of EO Major. The cells were grown in a humidified 37°C CO_2 incubator on poly-D-lysine coated plasticware in Eagles' Minimum Essential Media (E-MEM; Mediatech Inc., Herndon, VA, USA) supplemented with L-glutamine (3.0 mg/ml) and 10% heat inactivated fetal bovine serum (Mediatech, Inc., Herndon, VA, USA). The isolation of stromal cells from human tonsil has been described and these cells were a kind gift of MCG Monaco (Lisignoli *et al*, 1997; Monaco *et al*, 1996). These cells were maintained in RPMI-1640 media (Sigma, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum. The Jurkat T cell line and the Namalwa B cell line were obtained from ATCC and were maintained RPMI-1640 media supplemented with 10% heat inactivated fetal bovine serum. The human glial cell line, SVG, was established by transformation of human fetal glial cells by an origin defective SV40 mutant and has been previously described (Major *et al*, 1985). SVG cells were maintained in a humidified 37°C CO_2 incubator in E-MEM supplemented with L-glutamine and 10% heat inactivated fetal bovine serum. Peripheral blood mononuclear cells were collected from a laboratory volunteer by venipuncture and centrifuged over a layer Ficoll-Hypaque (Sigma). A hybrid JC Virus containing structural proteins from the Mad-1 genotype of JCV and regulatory sequences from SV40 was used in the virus binding assays. This virus has been extensively characterized and is useful for studying JCV receptor interactions (Liu *et al*, 1998; Vacante *et al*, 1989).

Virus purification and labeling

The preparation and labeling of JCV virions has been described (Liu *et al*, 1998a). in brief, 1×10^8 SVG cells were infected with 3200 hemagglutination units (HAU) of virus for 1 h at 37°C. At 3 weeks post-infection when the cells showed extensive cytopathic effect they were removed from the dishes by scraping and pelleted by centrifugation at 2000 r.p.m. for 30 min. The resulting cell pellet was suspended in 30 ml of the supernatant and subjected to three freeze-thaw cycles. Deoxycholic acid was then added to 0.25% and the suspension was incubated at 37°C for 1 h. Cell debris was removed by centrifugation at 5000 r.p.m. and the supernatants were layered on a cushion of cesium chloride (CsCl) (1.34 g/ml). Virus was banded by centrifugation for 24 h at 35 000 r.p.m. in a SW55Ti rotor. This virus band was removed and dialyzed extensively against phosphate buffered saline (PBS) (137 mM NaCl, 2.682 mM KCL, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2). Purified virus was stored in 100 ml aliquots at −80°C. Virus titers were determined by hemagglutination assay (HA). For virus labeling, 2.0 mg of gradient purified JCV was dialyzed overnight in labeling buffer (0.05 M Boric Acid, 0.2 M NaCl, pH 9.2). The virus was then incubated for 8 h at room temperature with 50 ml of a 5.0 mg/ml solution of fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA) dissolved in DMSO (Sigma, St. Louis, MO, USA) (Harlow and Lane, 1988). The FITC-labeled virus was purified by centrifugation over a cushion of cesium chloride. The FITC-labeled virus band was visualized with a hand-held UV light, removed, and dialyzed extensively against PBS (pH 7.2). The ratio of FITC to protein was determined by spectrophotometry.

Virus binding assays

Adherent cells were removed from culture dishes by incubation in Versene (0.15 M NaCl, 0.002 M KCl, 0.006 M Na_2HPO_4, 0.001 M KH_2PO_4, 0.001 M EDTA) and suspended at a concentration of 1×10^5 cells/ml in PBS. Non-adherent cells were washed in PBS and suspended at 1×10^5 cells/ml in PBS. The cells were incubated on ice with X HAU of FITC-

labeled JCV virions or with an equivalent amount (mg/ml) of FITC-labeled bovine serum albumin (BSA) as a negative control. After a 60 min incubation the cells were washed 1× in PBS containing 0.05 mg/ml propidium iodide, 2× in PBS, and fixed in 1.0 ml of PBS containing 1% paraformaldehyde. The cells were analyzed on a Becton-Dickinson FACScalibur™ flow cytometer using CellQuest™ software. Peripheral blood B cells were detected using a phycoerythrin (PE) conjugated antibody to CD19 and T cells were detected using a Quantum-red conjugated antibody to CD3.

Virus infectivity assays

5×10^5 PBMC or Namalwa B cells were incubated for 2 h at 37°C with 2048 HAU/ml of JCV. The cells were then pelleted, washed 3× in PBS, and incubated in media for 24 h. The cells were then pelleted and incubated for 10 min at 37°C with media or with Trypsin (to inactivate any virus remaining at the cell surface). The cells were then washed 3× in media containing 10% FCS (to quench the trypsin) and co-cultured for 7 days with 1×10^6 SVG cells. The SVG cells were then washed three times in PBS, removed from the dishes by incubation in Versene, and plated onto coverslips. The coverslips were then fixed in ice cold acetone and stained for the presence of JCV V antigen using a mouse monoclonal anti-SV40 V antigen antibody which is known to cross-react with JCV V antigen (PAB597). For the neuraminidase inhibition assays, Namalwa cells were treated with neuraminidase (*Vibrio cholerae*, 0.2 U/ml, Calbiochem-Novabiochem Corp, La Jolla, CA, USA) for 1 h at 37°C. The cells were then washed and incubated with 2048 HAU/ml of JCV for 1 h at 4°C. The cells were washed 3× in PBS and then incubated in growth media for 1 week. At 1 week post-infection untreated and neuraminidase treated Namalwa cells were lysed in 0.25% deoxycholate and the lysates clarified by centrifugation. The lysates did not contain detectable JCV by hemagglutination assay indicating that either no virus was produced from the infected cells or virus was produced at very low levels. We then diluted the lysates 1 : 20 in media and used this to inoculate SVG cells. SVG cells were then plated to coverslips and assayed for V antigen expression as described above.

RT–PCR amplification of JCV specific T and V antigen mRNA

Total RNA was isolated from 1×10^6 Namalwa B cells at 24 and 72 h post-infection using the S.N.A.P. total RNA isolation kit from InVitrogen. The RNA was then treated with RNAse-free Dnase for 15 min at room temperature and then inactivated by heating to 65°C for 15 min in the presence of EDTA. X micrograms of RNA was then reverse transcribed using the SuperScript cDNA synthesis kit from GIBCO BRL. The cDNA was then amplified using the following sets of primers. JCV T antigen primers: 5′ External: 4107–4126 5′-TGTCTACTTATAAAAGTTAC-3′; 3′ External: 4999–4979 5′-GAATAGGGAGGAATCCATG-3′; 5′ Internal: 4231–4252 5′-GGAATGCATGCAGATCTACAGG-3′; 3′ Internal: 4891–4872 5′ 5′-CCACCCTGATAAAGGTGGGG-3′. JCV V antigen primers: 5′ External: 1505–1526 5′-CCGTGCAAGTTCCAAAACTTTC-3′; 3′ External: 2500–2481 5′-GTCAACGTATCTCATCATGT-3′; 5′ Internal: 1991–2020 5′-GCCACAGTGCAATCTCAAGTCATGAACACA-3′; 3′ Internal: 2139–2121 5′-GGACATTTTCTCCTCCTG-3′.

The first round of PCR utilized external primer sets for T antigen and V antigen cDNA and consisted of 30 cycles of the following program in a 100 μl volume: 1 min at 95°C, 1 min at 55°C, 2 min at 72°C. This was followed by a 7 min extension period at 72°C. Ten μl of the first round of PCR was then amplified using internal primer sets for T antigen and V antigen cDNA using the same 30 cycles as for the first round. To determine the effect of neuraminidase on infection, Namalwa B cells were either untreated or treated with neuraminidase as described above. At 24 h post-infection total RNA was isolated, reverse transcribed to cDNA, and amplified with T antigen specific primers.

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