It has been suggested that JC virus (JCV) might travel to the central nervous system in infected B cells. Moreover, recent data suggest the presence of JCV in bone marrow plasma cells. However, the evidence for infection and replication of JCV in B cells is unclear. To address this question, we infected Epstein-Barr virus–transformed B cells with JCV and found that the viral genome decreased >1000-fold from days 0 to 20 after infection, which concurred with the absence of viral early and late messenger RNA transcripts and proteins. However, immunofluorescent images of B cells infected with fluorescein isothiocyanate–conjugated JCV demonstrated that JCV enters the B cells, and DNase protection assay confirmed the presence of intact JCV virions inside the B cells. Moreover, JCV-infected B cells were able to transmit infection to naive glial cells. These data confirm that JCV nonproductively infects B cells and possibly uses them as a vehicle for transmigration across the blood-brain barrier.

Progressive multifocal leukoencephalopathy (PML), a subacute demyelinating disease of the central nervous system [1] results from the lytic infection of oligodendrocytes, the myelin-producing cells in the brain, with human polyomavirus JC (JCV) [2–4]. JCV foci in a brain with PML are closely related to the blood vessels [5], and JCV is presumably spread by the hematogenous route from the primary site of infection to secondary sites, such as kidneys, lymphoid tissues, and brain, to establish focal areas of infection or persistence [2, 6–13]. However, the precise mechanism(s) of JCV dissemination throughout the body and trafficking across the blood-brain barrier remains poorly understood.

A possible role of B cells in JCV transmigration across the blood-brain barrier has been suggested [8, 12, 14–18]. JCV-infected B cells were first detected in the spleen and bone marrow of 2 patients with PML [15] and were subsequently detected in the central nervous system of a patient with PML [19]. Moreover, JCV DNA was reported to be associated with peripheral blood lymphocytes in 89.5% and 38% of patients with PML and patients with AIDS, respectively, with varying degrees of immunodeficiency [7, 10]. Demonstration of viral genome in the peripheral blood lymphocytes suggests the possibility of hematogenous spread of JCV. Furthermore, JCV infection of human B cells in vitro was suggested [12, 18, 20], and it was argued that JCV infection of Epstein-Barr virus (EBV)–transformed B cells or B cell lines resulted in viral DNA replication and production of infectious virions [18, 20]. However, evidence for JCV replication in B cells was inconclusive, because none of these studies quantitated the viral DNA, and messenger RNA (mRNA) transcripts were rarely detected [18]. Moreover, infectious JCV virions in the B cells might be the residual virus inoculum used...
to infect the B cells rather than de novo production of the virus in the B cells.

The possible role of B lymphocytes in JCV transmigration across the blood-brain barrier came to light again in 2005 after the development of PML in patients with multiple sclerosis and Crohn disease who were treated with a monoclonal antibody natalizumab (Tysabri; Biogen Idec and Elan Pharmaceuticals) [21–23]. Natalizumab is a recombinant humanized monoclonal antibody directed against the adhesion molecules α4β1 and α4β7 integrins [24]. Because α4β1 integrin interacts with very late antigen-4 (VLA-4) and this interaction is required for generating T and B cells from bone marrow progenitor cells in adult mice [25], it is argued that α4-integrin blockade mobilizes JCV-infected pre-B cells from bone marrow into the circulation and, thus, facilitates JCV dissemination and PML development [26, 27]. Recently, 3 patients treated with efalizumab for psoriasis also developed PML [28]. Efalizumab (Raptiva; Genentech) is a humanized monoclonal antibody (IgG1) that binds to the alpha chain (CD11a) of the leukocyte function associated antigen (LFA-1) [29, 30]. LFA-1 is a member of the heterodimeric β2 integrin family, and it interacts with intercellular adhesion molecules (ICAM) expressed on antigen presenting cells and endothelial cells and is necessary for T cell activation, T cell helper and B cell responses, natural killer cell cytotoxicity, and antibody-dependent cytotoxicity [30]. Efalizumab can selectively and reversibly block the activation, reactivation, and trafficking of T cells [29, 30]. Natalizumab and efalizumab both could create artificial cell-mediated immune deficiency in the central nervous system by inhibiting the migration of lymphocytes across the blood-brain barrier and, thus, may facilitate the development of PML.

To better understand the possible role of B cells in JCV transmigration across the blood-brain barrier, we studied the kinetics of JCV(Mad1) infection of B cells in vitro. Our data confirm that JCV nonproductively infects B cells. However, JCV virions persist in B cells, and B cells can transmit infectious virions to naive primary human fetal glial (PHFG) cells, suggesting that B cells can act as a vehicle for JCV transmigration across the blood-brain barrier.

**MATERIALS AND METHODS**

**Virus and cell cultures.** JCV(Mad1) was propagated in PHFG cells and was purified and quantitated by the HA assay and real-time polymerase chain reaction (PCR) [31–34]. EBV-transformed primary B cells were provided by Dr Allison Imrie, University of Hawaii, and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mmol/L L-glutamate at 37°C, with 5% CO₂ as described elsewhere [35, 36].

**JCV infection of EBV-transformed B cells.** EBV-transformed B cells (2.5 × 10⁶ cells) in suspension were infected with 250, 1000, or 2500 HA units of JCV(Mad1) for 2 h at 37°C, were washed with phosphate-buffered saline (PBS) 3 times, and aliquots of 2.5 × 10⁶ cells were harvested at days 0 (2 h), 5, 10, 15, and 20 after infection, for DNA and RNA extraction.

**Fluorescein isothiocyanate (FITC) labeling of JCV.** Sucrose-purified JCV [31] was labeled with FITC using the FluoroTag FITC Conjugation Kit (Sigma) by modifying the published protocol [37, 38]. Briefly, 850 μL of the sucrose-purified virus containing 100 HAU/μL of JCV was dialyzed overnight with labeling buffer (0.05 mol/L boric acid, 0.2 mol/L NaCl, pH 9.2), and the volume was adjusted to 1 mL. The dialyzed virus (1 mL) was then transferred into a 2-mL tube containing a small stirrer, and 250 μL of FITC solution (1 mg/mL) in 0.1 mol/L carbonate-bicarbonate buffer (pH 9.0) was added slowly drop by drop and incubated for 8 h at room temperature in the dark, with continuous gentle stirring. The FITC-labeled virus was then dialyzed overnight with PBS (pH 7.2), was aliquoted, and was stored at −80°C. As a control, 5 mg of bovine serum albumin (BSA) in 1 mL of PBS was also conjugated with FITC, was aliquoted, and was stored at −80°C. Protein and FITC levels in the FITC-conjugated viral or BSA suspension were determined according to the company’s protocol (Sigma) by measuring the absorbance at 280 nm and 495 nm, respectively. The FITC-conjugated BSA was diluted to get the same absorbance as FITC-conjugated JCV at 495 nm and was used as a nonspecific fluorescence control.

**Characterization of EBV-transformed B cell infection with FITC-conjugated JCV.** One million B cells were incubated with 1000 HAU of FITC-conjugated JCV (FITC-JCV) or FITC-conjugated BSA (FITC-BSA) containing the same amount of fluorescence in 500 μL of RPMI-1640 for 2 h. The B cells were washed 3 times with PBS, and the pellet was dissolved in 1 mL of PBS. Forty microliters of cell suspension containing ~40,000 B cells were transferred into each well of a multiwell slide, were air dried, and were fixed with 4% paraformaldehyde for 10 min. Cells were then washed with PBS and permeabilized with 0.4% Triton-X 100 for 5 min. The permeabilized cells were blocked with 5% BSA for 1 h and were incubated with anti-rabbit protein disulphide isomerase (1:500), to visualize the plasma membrane, and were washed 3 times with PBS. The cells were further incubated with anti-rabbit secondary antibody conjugated with Alexa-594 and were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Fluorescent cells were examined using an Axiocam MRm camera mounted on a Zeiss Axiovert 200 microscope, equipped with appropriate fluorescence filters and objectives as described elsewhere [32].

One million EBV-transformed B cells were incubated with 125, 250, 500, or 1000 HAU of FITC-JCV for 2 h and were washed with PBS. Cells were incubated with antibody against CD19 conjugated with PE (CD19-PE) and were subjected to
flow cytometry in Guava EasyCyte Plus platform. BSA labeled with FITC containing the same amount of fluorescence was used as a control.

**DNA and RNA extraction.** Uninfected and JCV-infected B cells in T25 flasks were washed with PBS, were counted, and an aliquot of 0.25 million cells in duplicate were either frozen for DNA extraction or lyzed with 350 μL of RLT plus buffer for RNA extraction (Qiagen) and stored at −80°C. RNA was isolated using protocols reported elsewhere [32]. Additionally, 100 μL of DNA was extracted using the Qiagen QIAprep Spin Miniprep Kit from each 0.25 million cells harvested at different time points, according to the manufacturer’s protocol.

**Quantitation of viral DNA and mRNA transcripts and reverse transcription PCR.** Two microliters of template DNA or complementary DNA were amplified and quantitated in the Bio-Rad’s iCycler iQ Multicolor Real-Time PCR Detection System with use of primers, probes, and protocols described elsewhere [39]. Copies of JCV TAg or VP-1 genomes or mRNA transcripts in experimental samples were calculated from the standard curve and expressed as copies of viral genome per 250,000 B cells or mRNA transcripts per microgram of total RNA [32, 39].

**RESULTS**

**JCV infection of B lymphocytes is nonproductive.** JCV VP-1 genome copies recovered from 2.5 × 10^5 infected B cells decreased >1000-fold from day 0 to day 20 after infection (Figure 1A) and neither early (TAg) nor late (VP-1) mRNA transcripts were detected (data not shown), suggesting that JCV infection of B cells is nonproductive. Our quantitative PCR and quantitative reverse transcription PCR assays were sensitive and consistently detected as low as 10–100 copies of JCV DNA and mRNA transcripts, respectively [31]. Because JCV infection of EBV-transformed B cells did not result in viral genome replication or transcripts expression, it is possible that JCV might have remained associated with the B cells by attaching to the cell membrane and might not have entered the B cells. To test this hypothesis, 2.5 × 10^5 B cells were inoculated with 250 HAU of JCV and incubated for 2 h at 37°C. After incubation, the cells were washed twice with PBS and trypsinized for 10 min with 0.5, 5, or 50 μg/mL of trypsin or with PBS alone, followed by 2 washes of PBS. Cells were cultured for up to 10 days. On days 1, 5, and 10, 2.5 × 10^4 trypsin-treated and untreated B cells were harvested, and JCV VP-1 DNA was amplified and quantitated by quantitative PCR. The data demonstrate that trypsin treatment had no significant effect in further reducing the JCV genome copies (Figure 1B), suggesting that JCV indeed entered into the B cells.

Moreover, by infecting EBV-transformed B cells with FITC-labeled JCV and by employing flow cytometry, we confirmed that JCV infects B cells in a dose-dependent manner. When B cells were incubated with 125, 250, 500, or 1000 HAU of JCV per million B cells, ~1.5%, 1.7%, 3.9%, or 7.7% of B cells, respectively, were infected with JCV (Figure 2A–2D).

We further characterized JCV infection of EBV-transformed B cells with use of FITC-labeled JCV. B cells and human brain microvascular endothelial (HBMVE) cells were either infected with FITC-labeled JCV (Figure 3E–3P) or inoculated with FITC-conjugated BSA (FITC-BSA) containing the same amount of fluorescence (Figure 3A–3D) and were examined by immunofluorescence microscopy. Interestingly, all FITC-labeled JCV virions were seen inside the HBMVE cells (Figure 3O–3P), whereas FITC-labeled JCV virions appeared to be inside the B cells and on the B cells surfaces (Figure 3G–3H and 3K–3L).
Figure 2. JC virus (JCV) infection of B cells is dose-dependent. One million Epstein-Barr virus–transformed B cells were incubated with 125 (A), 250 (B), 500 (C), or 1000 (D) HAU of fluorescein isothiocyanate (FITC)-JCV or with equivalent fluorescence-containing bovine serum albumin (A–D), similarly labeled with FITC (gray-filled histogram) as a control for 2 h, washed with phosphate-buffered saline, and subjected to flow cytometry.

These data confirm that JCV indeed infects the B cells. However, in contrast to HBMVE cells where VP-1 and T antigen proteins were observed predominantly in the cell nucleus [32], our repeated attempts to demonstrate T antigen and VP-1 proteins expression in the B cells failed, and it is unlikely that JCV infection of B cells is productive. Collectively, our data demonstrate that JCV infects the B cells; however, JCV infection of B cells is nonproductive.

Infectious JCV virions persist in the B cells. Because our data suggest that JCV infects EBV-transformed B cells but does not replicate in these cells, intact virus must survive inside the B cells long enough to be trafficked across the blood-brain barrier and must then have a mechanism to be released to infect susceptible cells, particularly the oligodendrocytes. However, our data suggest that JCV genome copy numbers rapidly decrease in the EBV-transformed B cells after infection, and it was unclear whether the decrease in viral genome was the result of degradation of viral DNA inside the B cells or merely a result of a dilution effect of replicating B cells, because in vitro EBV-transformed B cells replicate rapidly. To address this question, we infected 2.5 × 10⁶ EBV-transformed B cells with 250 HAU of JCV for 2 h. After a wash, 2.5 × 10⁵ cells were seeded in each well of a 24-well plate, were cultured, and all cells from each well were harvested at each time point. The cells were freeze-thawed 4 times to mechanically lyse the cells, and triplicate samples at each time point were either left untreated or treated with 125 U/mL of DNase for 1 h at 37°C to digest genomic and free viral DNA. The DNase-treated cells were heat inactivated at 75°C for 10 min, DNA was extracted from both DNase-treated and DNase-untreated cells, DNA concentration was measured, and JCV VP-1 DNA was quantitated by quantitative PCR. Figure 4A demonstrates that the total cellular DNA recovered from the B cells increased ~16-fold (mean ± standard deviation, 10.7 ± 1.5 to 170.9 ± 21.1 μg/mL) from days 0 to 15 after infection, suggesting that the B cells were actively replicating. However, mean JCV VP-1 DNA copies/well (± standard deviation) decreased ~11-fold (18.0 ± 8.6 × 10⁴ to 16.0 ± 7.5 × 10⁴ copies/well) from days 0 to 15 after infection, further confirming our observation that JCV infection of B cells is nonproductive. Although DNase treatment was very effective and reduced the total cellular DNA recovered from JCV-infected B cells, up to 96% (mean ± standard deviation, 170.9 ± 21.1 to 6.9 ± 1.7 μg/mL) on day 15 after infection (Figure 4A), DNase treatment had very little effect on the JCV VP-1 DNA copies recovered from the B cells (Figure 4B). These results suggest that JCV virions in the B cells remain intact for at least 15 days after infection and are protected from DNase digestion.

To further verify that JCV virions inside the B cells were infectious, JCV-infected B cells or lysates after infection of B cells with JCV for 24 h were further cocultured for an additional 24 h with naive PHFG cells. After 24 h coculture, the PHFG cells were washed; cells were harvested at days 5, 10, and 15 and were analyzed for viral late gene (VP-1) expression. Although no viral transcripts were recovered from PHFG cells cocultured with uninfected B cells (data not shown), JCV VP-1 mRNA transcripts increased >100-fold in PHFG cells cocultured with JCV-infected B cells or JCV-infected B cells lysate,
Figure 3. Epifluorescence microscopy demonstrating infection of B cells by fluorescein isothiocyanate (FITC)-conjugated JC virus (JCV). One million B cells were inoculated with either FITC-labeled bovine serum albumin (A–D) as a negative control or 1000 HAU of FITC-labeled JCV (E–L). Membranes were labeled with protein disulphide isomerase (PDI) and visualized with Alexa flour 594 (red). Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei (blue). Merged images demonstrate FITC-labeled JCV on the B cell surface (arrowheads) and inside the B cells (arrow) (H and L). M–P, Human brain microvascular endothelial cells infected with FITC-labeled JCV (positive control). Scale bar, 5 μm.
Figure 4. JC virus (JCV) genome in B cells was virion protected. Infected B cells harvested at different time points after infection were mechanically lysed by repeated freeze-thaw and were untreated or treated with DNase. DNA was extracted, concentration was measured, and JCV VP-1 DNA was quantitated by quantitative polymerase chain reaction. A, Total cellular DNA recovered increased from day 0 to day 15, suggesting that B cells were actively replicating and that DNase effectively reduced DNA concentration. B, JCV VP-1 DNA copies decreased from days 0 to 15 after infection as expected, but DNase treatment had very little effect on the viral DNA, suggesting that JCV genome in the B cells was virion protected from DNase digestion.

clearly indicating that the virions that remained inside the B cell cytoplasm were infectious and replicated efficiently in naive PHFG cells (Figure 5).

DISCUSSION

The mechanism of JCV trafficking across the blood-brain barrier remains poorly understood. PCR analyses have demonstrated that JCV may persist in the brain, tonsils, and lymphocytes of individuals with and without PML [2, 6–11, 13, 16, 40, 41], and it was proposed that JCV might employ B lymphocytes to cross the blood-brain barrier [8, 12, 14–16], similar to human immunodeficiency virus (HIV) and simian immunodeficiency virus, which gain entry into the brain via infected monocytes (Trojan horse) [42]. Further studies have also suggested the presence of JCV DNA in the peripheral blood mononuclear cells of 17.4% of 69 HIV type 1–infected immunocompetent patients, in 23.2% of 82 HIV type 1–infected immunocompromised patients, and in 60% of AIDS patients with PML [43]. However, there was no expression of JCV early and late mRNA transcripts in the peripheral blood mononuclear cells [43]. Recently, JCV DNA was demonstrated in the peripheral blood mononuclear cells and VP-1–stained CD138+ positive plasma cells were visualized in the bone marrow of a patient with rheumatoid arthritis treated with methotrexate, who developed PML and had a rapid fatal outcome, further suggesting that B lymphocytes may play an important role in the JCV latency and dissemination [14, 16]. Similarly, JCV DNA was detected in the bone marrow of HIV-negative and HIV–positive patients with and without PML, and JCV large T antigen but not VP1 was detected by double immunostaining in CD138+ plasma cells in an archival bone marrow specimen from an HIV-infected patient without PML [41]. Moreover, Focosi et al [44] detected JCV DNA in serial bone marrow samples from 4 hematological patients with histology-confirmed PML. Importantly, JCV DNA was first detected in the bone marrow and later in CSF, peripheral blood, and brain. Furthermore, in 1 patient who survived, JCV was not detected after resolution of PML, suggesting that JCV indeed disseminates by the hematogenous route [44].

It is often argued that JCV establishes low level of productive infection in B lymphocytes [8, 12, 18, 20]. Atwood et al [20] have demonstrated that 1% of B lymphocytes were JCV positive by in situ DNA hybridization on day 13 after infection, when
In an attempt to better understand the mechanism(s) of JCV transmigration across the blood-brain barrier, we previously demonstrated that JCV productively infects HBMVE cells, principal cells lining the blood-brain barrier, and proposed that cell-free JCV may cross the blood-brain barrier by infecting HBMVE cells [32]. Recently VP-1 expressing CD138+ plasma cells were demonstrated in the bone marrow with both rearranged and archetype regulatory regions, suggesting that B cells may latently harbor the virus and provide an environment for emergence of rearranged form of JCV, and thus, may act as a vehicle for JCV dissemination [16]. However, the relative role of B lymphocytes in JCV transmigration across the blood-brain barrier remains unclear. To address this issue, we employed EBV-transformed B cells and studied the replication kinetics of JCV. Although we did not find any evidence of JCV replication in EBV-transformed B cells in vitro, we observed that JCV nonproductively infects EBV-transformed B cells and JCV virions remain intact inside the B cells, presumably long enough to employ B cells as a potential vehicle to cross the blood-brain barrier and transmit infection to the oligodendrocytes in the brain.

Our study does not preclude the possibility that primary B cells, CD34+ hematopoietic precursor cells, or other cells within this lineage would not support JCV replication. In fact, JCV infection of primary hematopoietic CD34+ cells as well as hematopoietic progenitor cell lines KG-1 and KG-1a has been suggested [9, 12]. However, there are no studies comparing JCV replication between primary and EBV-transformed B cells; interestingly, our data on EBV-transformed B cells are consistent with earlier studies that documented low efficiency of JCV infection of primary B cells and hematopoietic precursor cells [9, 12]. Moreover, JCV T antigen– and VP-1–expressing primary B cells decreased from 1%–3% on day 1 to 0.5% on day 8 after infection [9], suggesting that productive JCV replication is unlikely to occur even in primary B cells and hematopoietic precursor cells. These studies used less sensitive immunostaining, HA assay, or standard PCR to determine JCV infection.

Although we always detected JCV TAg and VP-1 DNA, viral mRNA transcripts were never detected in JCV-infected B cells, suggesting that JCV transcripts expression was either extremely low or completely shut off in the B cells. Further in vitro and in vivo studies using sensitive state-of-the-art real-time PCR and quantitative reverse transcription PCR assays are warranted to define the role of hematopoietic precursor cells, primary B cells, and bone marrow–derived plasma cells in JCV replication, latency, and dissemination.

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