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JC Virus Infection of Hematopoietic Progenitor Cells, Primary B Lymphocytes, and Tonsillar Stromal Cells: Implications for Viral Latency

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The human polyomavirus JC virus (JCV) infects myelin-producing cells in the central nervous system, resulting in the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). JCV-induced PML occurs most frequently in immunosuppressed individuals, with the highest incidence in human immunodeficiency type 1-infected patients, ranging between 4 and 6% of all AIDS cases. Although JCV targets a highly specialized cell in the central nervous system, infection is widespread, with more than 80% of the human population worldwide demonstrating serum antibodies. A number of clinical and laboratory studies have now linked the pathogenesis of PML with JCV infection in lymphoid cells. For example, JCV-infected lymphocytes have been suggested as possible carriers of virus to the brain following reactivation of a latent infection in lymphoid tissues. To further define the cellular tropism associated with JCV, we have attempted to infect immune system cells, including $CD34^+$ hematopoietic progenitor cells derived from human fetal liver, primary human B lymphocytes, and human tonsillar stromal cells. Our results demonstrate that these cell types as well as a $CD34^+$ human cell line, KG-1a, are susceptible to JCV infection. JCV cannot, however, infect KG-1, a $CD34^+$ cell line which differentiates into a macrophage-like cell when treated with phorbol esters. In addition, peripheral blood B lymphocytes isolated by flow cytometry from a PML patient demonstrate JCV infection. These results provide direct evidence that JCV is not strictly neurotropic but can infect $CD34^+$ hematopoietic progenitor cells and those cells which have differentiated into a lymphocytic, but not monocytic, lineage.

JC virus (JCV), a DNA virus of the *Papovaviridae* family, is the etiologic agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disorder of the central nervous system associated with compromised immunity. On the basis of the detection of JCV-specific antibodies, it is estimated that more than 80% of the human population has been infected by JCV (23, 34). Seroconversion generally occurs during childhood, since 65% of adolescents produce JCV-specific antibodies (23). Although primary infection appears to be asymptomatic, JCV may persist in a latent state for an extended period, perhaps for life, even in immunocompetent individuals. JCV reactivation, however, may occur if immune function is compromised.

Reactivation of JCV, with concomitant development of PML, has been found in immunosuppressed patients receiving bone marrow or renal transplants (5, 15). JCV viruria is detected in bone marrow transplant patients (5), but only in those who have tested positive for JCV antibodies by enzyme-linked immunosorbent assay prior to immunosuppression. Thus, viruria most likely results from a JCV infection which occurred prior to the time of immunosuppression (5). Moreover, PML patients have immunoglobulin G antibodies to JCV even though their immunity is compromised, providing further evidence that PML results from reactivation of latent JCV infection. Neither the cell types harboring JCV nor the sites of its reactivation from latency have been well characterized.

Previously, B-cell lines were shown to be susceptible to JCV infection in vitro (6). We have also found JCV DNA-associated kappa-light-chain-positive lymphocytes from bone marrow and spleens of two PML patients (18). Additionally, using PCR assays, we and others have amplified JCV-specific sequences from peripheral blood lymphocytes obtained from both PML (31) and immunosuppressed (11, 28) patients. These findings clearly demonstrate that cells of the peripheral lymphoid system carry the JCV genome. In the event of either induced or acquired immunodeficiency, these latently infected cells would potentially initiate JCV replication, with the release of viral particles.

It remains unclear whether peripheral B cells are the principal targets of early JCV infection in lymphoid tissue or whether other cells within the hematopoietic microenvironment may also serve as potential virus reservoirs. In particular, it is of interest to determine if early hematopoietic progenitor ($CD34^+$) cells and the stromal elements with which lymphoid cells interact are also susceptible to infection. Accordingly, we have compared the in vitro susceptibilities of human hematopoietic progenitor ($CD34^+$) cells, primary B lymphocytes, and human tonsillar stromal cells to JCV infection. These studies provide a better understanding of the cellular tropism of JCV and identify additional viral targets that may contribute to clinical viremia and the mechanisms of PML induction.

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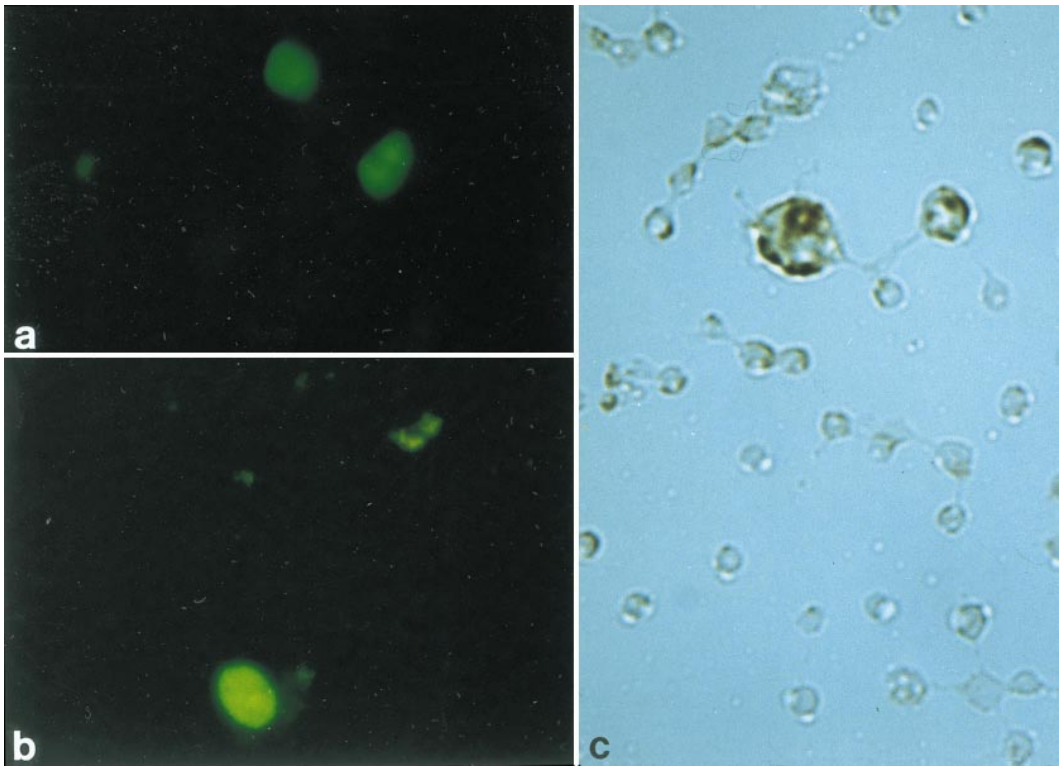

FIG. 1. JCV infection of KG-1a, a human hematopoietic cell line. The infected KG-1a cells were by immunofluorescence examined 5 days postinfection. T-antigen antibody PAB 416 (a) and V-antigen antibody PAB 597 (b) were used for detection. Positive cells were visualized with a secondary goat anti-mouse fluorescein isothiocyanate-conjugated antibody. (c) JCV-infected KG-1a cells were examined by in situ hybridization with a JCV biotin-labeled DNA probe. Magnification, × 40.

MATERIALS AND METHODS

Hematopoietic progenitor cells. Hematopoietic $CD34^+$ cell lines (KG-1 and KG-1a), originating from bone marrow of an acute myelogenous leukemia patient, were obtained from the American Type Culture Collection, Rockville, Md. KG-1a cells, which are morphologically, cytochemically, and functionally less mature than the parental KG-1 cells (20), were propagated in RPMI 1640 with 20% fetal bovine serum. The parental KG-1 line, when cultured with phorbol 12-myristate 13-acetate (PMA) (19), differentiated into a more mature macrophage-like state. For this reason, KG-1 cells were cultured with 10^{-7} M PMA for 3 days prior to use.

Primary $CD34^+$ progenitor cells were collected from human fetal liver at 16 to 18 weeks of gestation. To separate mononuclear cells from hepatocytes, the liver tissue was fragmented and passed through sieves (Thomas Scientific, Swedesboro, N.J.) of different pore sizes (3 in. [7.62 cm], 60 mesh, and 30 mesh). The mononuclear cells were recovered after stratification on a Ficoll-Hypaque density gradient (M.A. Bioproducts, Walkersville, Md.) and washed twice in RPMI 1640–10% fetal bovine serum. Mononuclear cells (2×10^7 in a 25-cm^2 flask coated with anti-CD34 antibody; Applied Immune Sciences Inc., Santa Clara, Calif.) were then incubated for 1 h at room temperature. The nonadherent cells were discarded, and the adherent $CD34^+$ cells were dissociated from the flask by agitation. Both adherent and nonadherent cells were analyzed by flow cytometry (FACSort; Becton Dickinson, Mansfield, Mass.). The adherent cells were labeled with a panel of monoclonal antibodies including CD2, CD3, CD10, CD13, CD14, CD15, CD19, CD33, CD34, CD38, and CD45 (Becton Dickinson, San Jose, Calif.) to test their purity. The adherent population, containing >96% $CD34^+$ cells, was used for in vitro infection experiments.

Primary B cells. Mononuclear cells were isolated from tonsils of patients (4 to 20 years old) after tonsillectomy or from peripheral blood of normal donors by centrifugation on Ficoll-Hypaque gradients. T lymphocytes were removed from the mononuclear preparations by rosetting with sheep erythrocytes treated with aminoethylisothio-uronium bromide (Sigma, St. Louis, Mo.) (7), and the B lymphocyte population was recovered. The percentage of B cells was determined by fluorescence-activated cell sorting (FACS) analysis. Only cell preparations having >95% CD19 cells were used in the experiments. The purity of these preparations was further tested with anti-CD3, anti-CD4, anti-CD8, and anti-CD14 monoclonal antibodies (Becton Dickinson).

Human primary stromal cells. The method used to obtain human tonsillar stromal cells has previously been described in detail (21). Briefly, human tonsils were obtained from 20 tonsillectomized children ranging in age from 3 to 8 years. Tonsillar parenchyma was separated from tissue, dissected into small pieces, and placed in 20 ml of calcium- and magnesium-free Hanks saline solution containing 150 U of collagenase (GIBCO BRL, Grand Island, N.Y.) per ml, 0.1% trypsin (ICN Biochemicals, Cleveland, Ohio), and 2% heat-inactivated chicken serum (GIBCO BRL). The tissue was then incubated in a water bath for 1 h at 37°C. After enzymatic digestion, the tissue pieces and cells were washed twice with RPMI 1640 with 10% fetal bovine serum, resuspended in medium, and seeded into T75 flasks (Costar, Cambridge, Mass.). Nonadherent cells were subsequently removed, and the adherent stromal cells were expanded by several passages. The medium was changed twice weekly.

In vitro JCV infection. The following nonadherent human cells, primary $CD34^+$ hematopoietic precursors, primary B lymphocytes from tonsils or peripheral blood, and cells of the line KG-1, KG-1a, or U937 were each seeded at 10^5 cells per well in six-well plates. Adherent stromal cells were also seeded at the same density into similar vessels containing glass coverslips. All cell types were inoculated with 300 hemagglutination (HA) units (HAU) of JCV (MAD-4 strain). Two hours after infection, the cells were washed and new medium was added to the cultures. Five days later, the cells were harvested and fixed for in situ hybridization and immunofluorescence analysis. Human fetal glial (HFG) cells were used as a positive control of JCV infection.

Immunofluorescence staining for T and V viral antigens. Detection of JCV T antigen was considered a measure of early viral transcription, while detection of V antigen (capsid protein) denoted late viral transcription and potential virion formation.

After harvesting, all infected nonadherent cell types were washed twice in phosphate-buffered saline (PBS), resuspended at 10^6 cells per ml, dried onto glass coverslips, and fixed for 15 min at −20°C with 100% acetone and then with 100% methanol. Adherent stromal cell cultures on coverslips were similarly fixed. After rehydration of cells in PBS, all cell types were treated with an antibody (PAB 416; Oncogene Science, Inc., Cambridge, Mass.) to simian virus 40 T antigen diluted 1:10 in PBS. This antibody cross-reacts with the amino-terminal sequence of the JCV T antigen (23). Cells were also tested for the presence of V antigen by treatment with an antibody to simian virus 40 capsid protein, which also cross-reacts with JCV capsid proteins (PAB 597; a gift from L. Norkin, University of Massachusetts, Amherst), diluted 1:500 in PBS. After 30 min of incubation at room temperature, the coverslips were washed twice in PBS and incubated for 30 min at room temperature in the dark with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson Immunoresearch, West Grove, Pa.) diluted 1:50 in PBS. The coverslips were washed twice in PBS and mounted on glass slides with 2% antifade (1,4-diazabicyclo-[2.2.2.]octane; Sigma) in 90% glycerol. By using a Zeiss ICM 405 epifluorescence

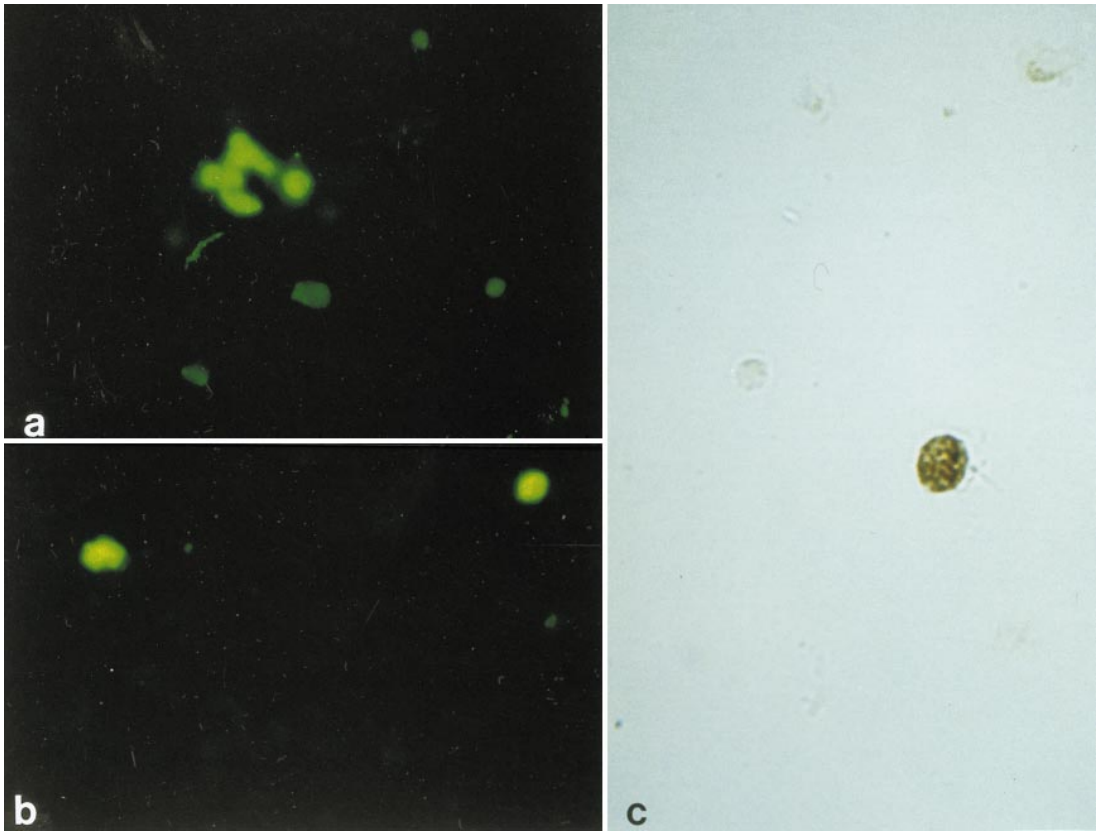

FIG. 2. Primary $CD34^+$ cells infected in vitro with JCV. Five days postinfection, the expression of T and V antigens was detected by indirect immunofluorescence. (a) Representative field of $CD34^+$ progenitor cells positive for viral T protein; (b) cells positive for JCV V antigen. (c) In situ hybridization with a biotinylated DNA probe for JCV genome was performed in primary human $CD34^+$ cells 5 days postinfection. A single cell in the lower center of the field contains JCV DNA, as demonstrated by the intranuclear brown reaction product (no hematoxylin counterstain). Magnification, ×40.

microscope, the percentage of T- and V-antigen-positive cells was calculated from 25 different fields from each experiment.

In situ hybridization. A positive signal in the in situ hybridization assay indicates de novo viral DNA replication (1, 2). Replication of JCV DNA was analyzed by in situ DNA hybridization in all cell types, using a full-length JCV biotinylated DNA probe (ENZO Diagnostic Inc., Farmingdale, N.Y.). The in situ DNA hybridization protocol used was described previously (17). This method does not require protease digestion and acid hydrolysis. These steps are not necessary for entry of the biotin-labeled DNA probe into lymphocytes dried on coverslips.

The hybridization reaction mixture contained 50% (vol/vol) deionized formamide, 10% dextran sulfate, 0.4 μg of herring sperm DNA per ml, and 2 ng of biotinylated probe DNA per ml in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A 25-μl aliquot of this mixture was applied to the coverslips; a larger coverslip was placed on top, and the edges were sealed with rubber cement. Both the cells and the DNA probe were denatured in an oven set at 85°C for 10 min, and hybridization was performed at 37°C for 48 h. The hybridization signal was detected with a streptavidin-biotin-horseradish peroxidase kit (Detek I-hrp; ENZO Diagnostic). Diaminobenzidine tetrahydrochloride was used as the chromogen.

Assay of HFG cell culture harvests for infectious JCV. Harvests of supernatant medium were made from all cultures 1 week after inoculation with JCV. A small aliquot was used to measure JCV replication by HA of human type O erythrocytes (26, 35, 36). HA was used to measure production of JC virions by the various cell types studied after passage onto highly susceptible human fetal brain cultures. The remainder of the harvests were filtered and added to cultures of uninfected primary HFG cells to determine whether infectious JCV was contained in the harvests. Glial cell cultures were harvested 5 days after inoculation and assayed by both in situ hybridization and immunofluorescence analysis (as described above).

Flow cytometry from in vivo lymphocytes. Peripheral blood mononuclear cells (PBMC) from two AIDS patients were examined for the presence of the JCV genome. One patient was clinically diagnosed with PML. This diagnosis was confirmed by histopathology of brain biopsy specimens. In situ hybridization of these brain biopsy specimens with a JCV probe also confirmed the diagnosis. The second patient did not have PML. Blood (20 ml) was collected in heparinized tubes from each patient by venipuncture. PBMC were isolated from each blood sample by centrifugation on Ficoll-Hypaque gradients by a previously described protocol (31). Cells were counted, adjusted to 10^6/ml in PBS, and incubated for 30 min on ice with 200 μl of Simultest B- and T-cell reagent containing anti-CD3 and anti-CD19 antibodies in the same tube (Becton Dickinson) or with the Simultest negative control antibodies (Becton Dickinson) diluted 1:5 in PBS. The cells were then washed twice in cold PBS and once in sheath fluid (Baxter Diagnostics Inc., Deerfield, Ill.) and fixed in 1 ml of sheath fluid containing 1% paraformaldehyde. Equal numbers of B and T cells were collected from each patient by cell sorting into 50-ml Falcon tubes. PBMC from a JCV-negative individual were also stained and sorted by the same protocol to control for possible virus contamination of the cell sorter. After sorting, the cells were pelleted and a fraction was reanalyzed on the flow cytometer to assess purity. The remaining sorted cells and an equivalent number of unsorted PBMC from these patients were lysed in 0.5 ml of 10% sodium dodecyl sulfate and 55 μl of proteinase K (10 μg/μl). DNA from lysed cells was extracted twice with phenol, twice with a 1:1 mixture of phenol-chloroform, and twice with a 29:1 mixture of chloroform-isoamyl alcohol and precipitated in 2.5 volumes of cold ethanol containing 0.1 volume of 3 M sodium acetate.

Nested PCR and Southern analysis. Nested PCR was performed with primers from the T-antigen coding region of the JCV genome. Primers were purchased from GIBCO BRL (Gaithersburg, Md.). The primer sites and their locations within JCV genome were as follows. The external primer set was 5′-GAATAG GGAGGAATCCATGG-3′ (nucleotides [nt] 4999 to 4979) and 5′-GGAATGC ATGCAGATCTACAGG-3′ (nt 4231 to 4252). The internal primer set was: 5′-GGTGGGGGACGAAGACAAGATG-3′ (nt 4878 to 4858) and 5′-GTGTTG GGATCCTGTGTTTTC-3′ (nt 4301 to 4321).

PCRs were performed in a total volume of 100 μl with 2.5 U of *Taq* polymerase and 2 mM $MgCl_2$ as instructed by the manufacturer (Perkin-Elmer, Branchburg, N.J.). After an initial period of 5 min at 95°C, reaction products were amplified for 30 cycles, using the following program: 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. Amplification was completed with a 7-min extension period at 72°C. One positive and two negative controls were included in all PCR experiments. Negative controls consisted of the reaction mixtures with no DNA template or 1 μg of B-cell DNA from a JCV-negative individual. The positive control was 1 ng of DNA from the JCV plasmid pM1Tc.

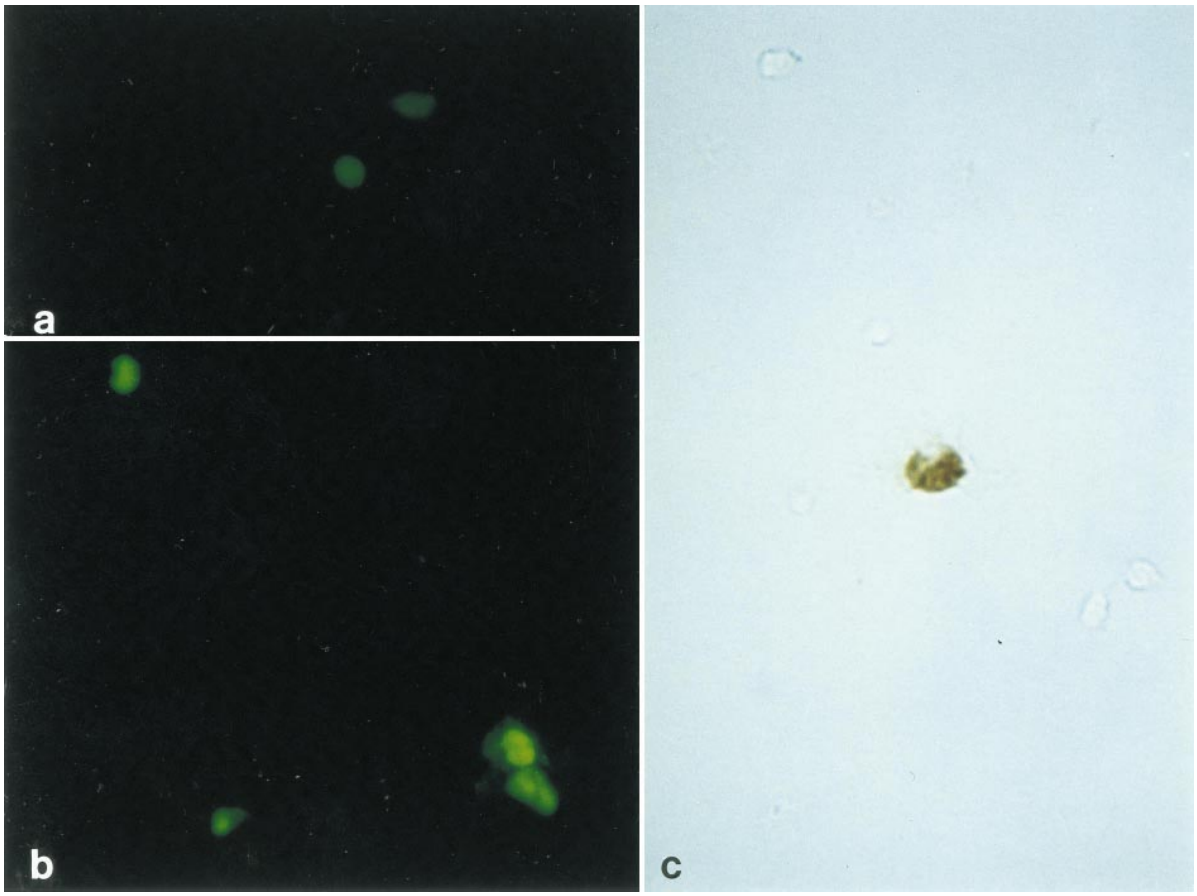

FIG. 3. Primary human tonsillar B lymphocytes infected in vitro with JCV. Immunofluorescence analysis was performed to detect T (a) and V (b) viral proteins. Five days postinfection, JCV-positive B lymphocytes were detected by in situ hybridization (c) with a biotinylated JCV probe. Cells positive for JCV have a brown precipitate in their nuclei, as seen in the B lymphocyte in the center of the field (no hematoxylin counterstain). Magnification, ×40.

The reaction mixture with external primers contained 1 μg of cellular DNA. Nested PCR was performed with 10 μl of the reaction product from the first amplification.

Southern blot analysis was performed with 20 μl of reaction mixture from the nested primer amplification as previously described (31).

RESULTS

Human CD34$^+$ cell infection with JCV. Previous clinical and cell culture data suggest that JCV can infect lymphoid cells (6, 11, 18, 31), particularly those cells expressing the B-cell phenotype (6, 18). If lymphoid tissue is an initial site of limited infection and also a potential site for viral latency, then human hematopoietic precursor cells may also be susceptible to JCV infection. Although human stem cells do not express cell surface markers associated with lineage cell commitment (4, 8, 24, 32), they do express the CD34 antigen, which is a stem cell marker (4, 8, 24, 30, 32, 33).

Two cell lines, KG-1 and KG-1a, are available for studying hematopoietic progenitors and their differentiation. The KG-1 cell line was derived from a patient who presented with erythroleukemia but eventually developed acute myelogenous leukemia (20). Both KG-1 and KG-1a cells exhibit the CD34 antigen marker on cytofluorographic analysis. After treatment with phorbol esters, KG-1 cells differentiate into mature macrophages (19), whereas KG-1a cells are unaffected by this treatment. The KG-1a cell line is composed of undifferentiated blast cells, which are morphologically, cytochemically, and functionally less mature than the KG-1 cells (20). For these reasons, untreated KG-1 and KG-1a cells and PMA-treated KG-1 cells were inoculated with JCV to determine their in vitro susceptibility to infection.

Untreated KG-1 and KG-1a cells were infected by JCV, as demonstrated by both immunocytochemistry and in situ hybridization (Fig. 1). However, when parental human KG-1 cells were treated with PMA and differentiated into cells with macrophage-like characteristics, they were no longer susceptible. Immunocytochemistry and in situ hybridization assays also indicated that the promonocytic cell line U937 was not susceptible to JCV infection (data not shown). These data provide evidence that cells of monocytic lineage are not susceptible to JCV infection in vitro. The susceptibility of the less mature KG-1a cells to JCV infection prompted us to test the susceptibility of primary CD34$^+$ cells. Therefore, CD34$^+$ cells were isolated from human fetal liver, the main hematopoietic organ in the fetus; an immunofluorescence assay was used to test their susceptibility to JCV infection. T (Fig. 2a) and V (Fig. 2b) antigens were detected in primary CD34$^+$ cells 5 days after in vitro infection with JCV. JCV DNA in primary CD34$^+$ cells was also detected by in situ hybridization (Fig. 2c).

JCV infection of primary human B lymphocytes. Previously we demonstrated that cells of B-cell lineage (18) and B-lymphoblastoid cell lines (6) were susceptible to JCV infection. We have now extended this work by testing the susceptibility of primary B lymphocytes from peripheral blood and tonsillar

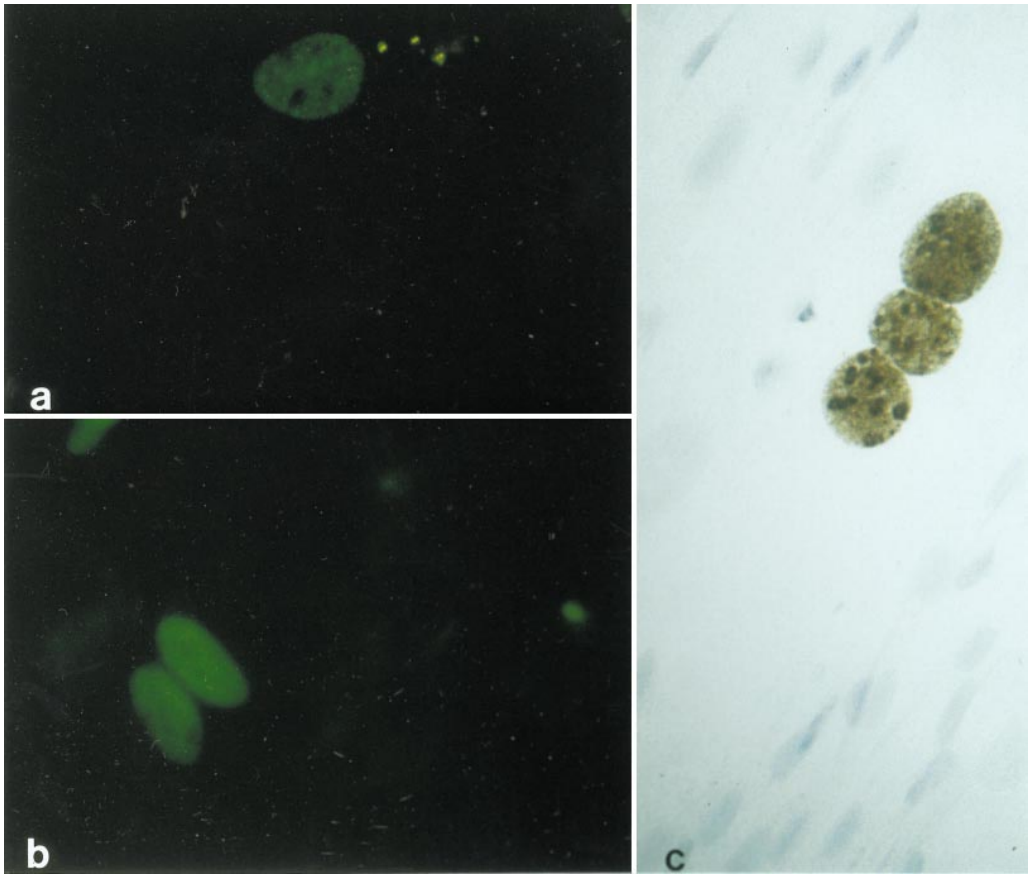

FIG. 4. In vitro infection of human tonsillar stromal cells. (a) Representative field of viral T-antigen-positive cells; (b) representative field of viral V-antigen-positive cells. (c) In situ hybridization was performed with a biotinylated DNA probe for the JCV genome. Positive intranuclear staining (brown reaction product) appears in three cells in the center of the field (hematoxylin counterstain). Magnification, ×16.

tissue of normal donors (as described in Materials and Methods) to JCV infection. We used only cells found to be $CD19^+$ by flow cytometric analysis. Five days after inoculation with JCV, primary tonsil and peripheral B lymphocytes were tested by immunofluorescence for the presence of viral T and V proteins. As shown in Fig. 3a and b, primary B cells were positive for JCV T and V proteins. B lymphocytes from peripheral blood or tonsil were positive for JCV DNA when tested by in situ hybridization with a biotin-labeled JCV DNA probe. JCV DNA was found in approximately 2% of the human B lymphocytes studied (Fig. 3c).

JCV infection of primary human stromal cells. Stromal cells contribute to the architecture and function of lymphoid organs and are in direct contact with circulating B lymphocytes. For this reason, stromal cells were tested for the capacity to support JCV replication and ability to serve as a reservoir for the infection of other cell types with which they interact. Stromal cell cultures were inoculated with JCV and 5 days later were monitored by immunofluorescence analysis. In these cultures, T antigen was detected in 25% of the cells (Fig. 4a) and V antigen was detected in 10% (Fig. 4b). Furthermore, primary human stromal cells were shown by in situ hybridization to be JCV DNA positive (Fig. 4c).

In Table 1, we compared the percentage of immune system cells positive for both JC viral proteins and JCV DNA with the percentage of control HFG cells. Glial cells are currently recognized as the most susceptible to JCV infection. The percentage of stromal cells positive for T and V antigens and viral DNA was only 2.5 times lower than the percentage of HFG cells. However, the percentage of $CD34^+$ cells positive for JCV was about 15 times lower than the percentages of HFG cells as well as primary human B lymphocytes.

Virion production from KG-1a, $CD34^+$, and B cells. Although we have demonstrated the susceptibility of KG-1a cells, $CD34^+$ cells, and B lymphocytes to JCV infection, the percentage of JCV-infected cells was low. Furthermore, only about 4 HAU of JCV per 50 μl was present in the supernatant medium of infected KG-1a cells, primary $CD34^+$ cells, and B lymphocytes. To verify that infectious virus was produced by these cell types, cell lysates of infected cultures were inoculated onto highly susceptible HFG cells. After inoculation of the HFG cells with the cell lysate inoculum, the HA titer increased to 2,048 HAU/50 μl. In situ hybridization also confirmed the HA results (Fig. 5). Conversely, the cell lysate inoculum from KG-1 PMA-treated cells and the U937 cell line was not able to infect HFG cells (data not shown).

B lymphocytes from PML patients contain JCV DNA. Our in vitro experiments show that $CD19^+$ lymphocytes are infected by JCV but to a much lower degree than HFG cells. These results might be expected if such cells serve as a site of JCV latency. Detection of JCV DNA in peripheral lymphocytes of immunosuppressed patients also supports our findings (11, 28, 31). Since we found that different B-cell lines are also susceptible to JCV infection (6), we evaluated which lymphocyte subpopulation carried the JCV genome.

PBMC from a non-PML patient (patient 1) and a PML

TABLE 1. Detection of JCV products in immune system cells

Sample[a]	Mean of 5 expts ± SD			
	T-antigen-positive cells[b] (%)	V-antigen-positive cells[b] (%)	Cells replicating viral DNA[c] (%)	Viral multiplication (HAU/50 μl)[d]
HFG cells	60.2 ± 8.5	25.3 ± 5.4	15.4 ± 2.3	4,096
Human tonsillar stromal cells	25.3 ± 3.3	10.1 ± 2.4	5.9 ± 1.5	1,024
Primary $CD34^+$ cells	5.2 ± 0.8	3.6 ± 0.4	1.2 ± 0.4	<4[e]
KG-1a cells	3.1 ± 0.5	3.4 ± 0.4	1.7 ± 0.6	<4[e]
Primary tonsillar B lymphocytes	4.1 ± 0.7	3.1 ± 0.6	1.5 ± 0.5	<4[e]

[a] All cell types were infected in vitro with 300 HAU of JCV (MAD-4 strain) per ml and cultivated for 5 days.
[b] Immunofluorescence detection of viral antigens.
[c] Replication of viral DNA was determined with a biotin-labeled JCV DNA probe and in situ DNA hybridization (17).
[d] Hemagglutination of human type O erythrocytes.
[e] Supernatants from initial infection were used as an inoculum on the more susceptible HFG cells (Fig. 5).

patient (patient 2) were stained with monoclonal antibodies to CD3 antigen for T cells and CD19 antigen for B cells. The stained B- and T-cell populations were then sorted, as described in Materials and Methods, with a FACSort flow cytometer to >99% purity (Fig. 6). DNA was extracted from the unsorted and sorted cell populations and subjected to nested PCR. JCV DNA was detected only in the unsorted PBMC population and the sorted B-cell subpopulation of the PML patient (Fig. 7). There was no JCV DNA in either the T-cell population from the PML patient or the T-cell, B-cell, or unsorted populations from the non-PML patient. Lymphocytes which were sorted to control for possible contamination of the cell-sorting apparatus were negative by PCR for JCV DNA (Fig. 7).

DISCUSSION

The human polyomavirus JCV is responsible for the demyelinating disease PML in the human brain. It is a lytic infection of oligodendrocytes, which are myelin-producing differentiated cells. While PML occurs in patients who are immunocompromised, primary exposure to JCV is not associated with any known clinical manifestations. JCV was detected in the kidneys of healthy individuals after primary infection, suggesting that

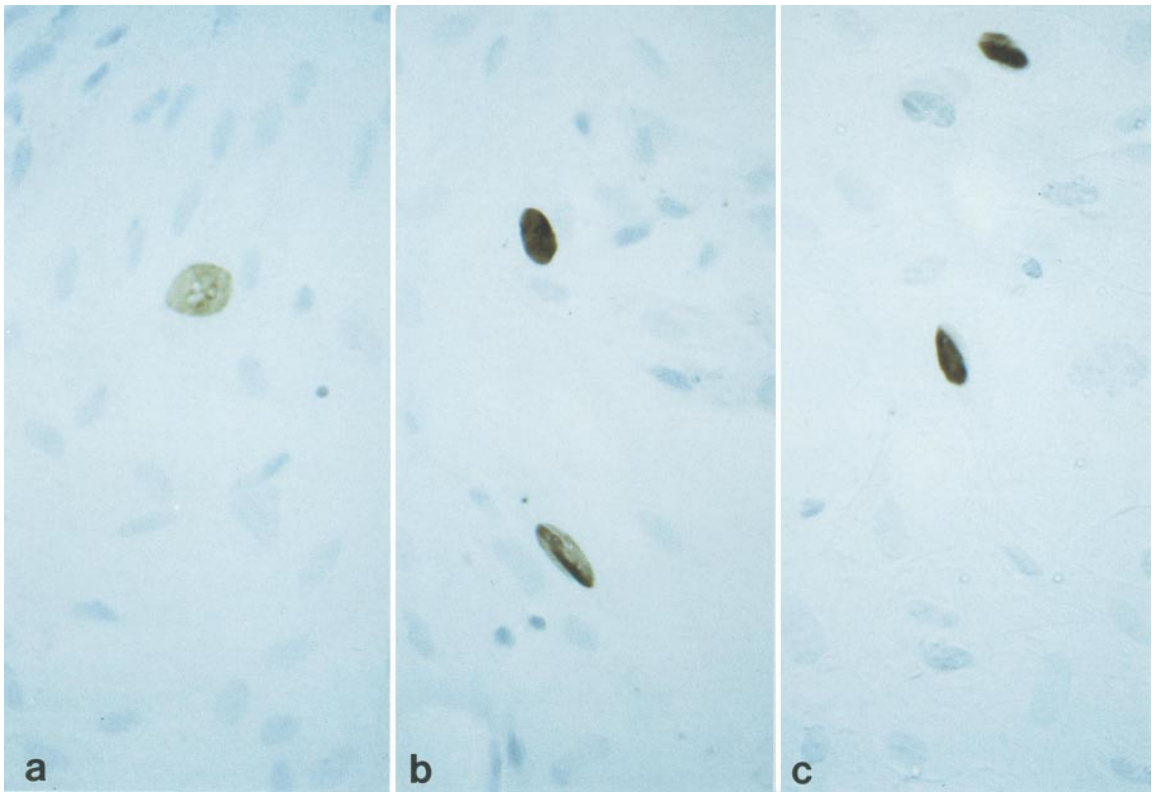

FIG. 5. HFG cells infected with supernatants from infected $CD34^+$ progenitor cells (a), KG-1a cells (b), and B lymphocytes (c). $CD34^+$ cells, KG-1a cells, and B lymphocytes were infected in vitro with 300 HAU of JCV (Mad-4 strain) per ml, and the supernatants were collected, filtered, and added to fresh cultures of uninfected HFG cells as described in Materials and Methods. After 5 days, the inoculated cultures of HFG cells were harvested for in situ DNA hybridization analysis. JCV-positive cells show a brown precipitate. Cells were subsequently counterstained with hematoxylin. Magnification, ×16.

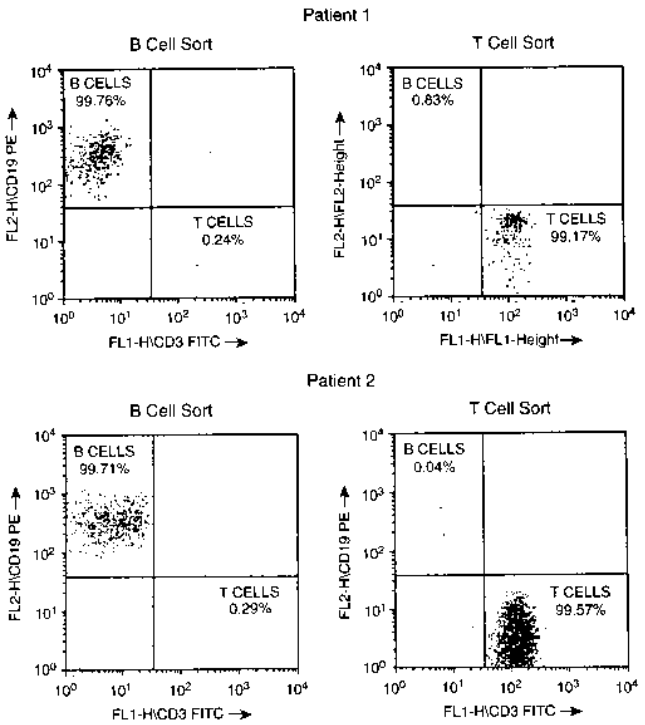

FIG. 6. Postsort FACS analysis of $CD19^+$ B cells and $CD3^+$ T cells from an AIDS patient without PML (patient 1) and an AIDS patient with PML (patient 2). $CD19^+$ B cells were sorted to 99.76% purity from patient 1 and 99.71% purity from patient 2. $CD3^+$ T cells were sorted to 99.17% purity from patient 1 and 99.57% purity from patient 2.

this virus may establish a latent infection in renal tissue (10). Several reports have also shown that lymphocytes may be the site of primary infection and subsequent virus latency (11, 18, 28). Virus reactivation can result in cytolytic invasion of the nervous system, which occurs most often in immunosuppressed individuals. However, the site of viral reactivation from latency is unknown.

To gain insight into the range of cell types susceptible to JCV and their potential roles in latency and reactivation, we tested the susceptibility to JCV infection in primary $CD34^+$ hematopoietic progenitor cells, the $CD34^+$ cell lines KG-1 and KG-1a, primary B lymphocytes, and stromal cells of the tonsil. Our results show that all cells are infectable by JCV. The viral T protein, the first protein to be expressed, plays a critical role in both viral replication and late structural protein (V antigen) expression. Our data show that in both $CD34^+$ and B cells, the T and V viral proteins are present, although at low levels.

The low percentage of B lymphocytes infected by JCV in vitro correlates with the in vivo data. Dorries et al. (11) reported that the concentration of virus-specific DNA in peripheral blood lymphocytes of immunocompetent individuals was low, estimated in the range of less than one viral genome equivalent per 20 cells, compared with thousands of copies of the JCV genome in cytolytically infected CNS tissue of PML patients. This observation raises the possibility that only a specific subpopulation of B lymphocytes is susceptible to JCV infection. We have begun to address the question of the susceptibility of putative subpopulations of B lymphocytes by use of flow cytometry and cell sorting analysis. However, the observation that the relatively homogeneous B-cell lines BJA-B and Namalwa show only limited susceptibility to JCV infection suggests that a low-level infection occurs in B lymphocytes. However, in vivo studies have reported a high incidence of polyomavirus DNA in lymphoid cells in leukemic patients, both before and after bone marrow transplantation (28).

Our results are analogous to the studies of other viruses that are carried in lymphocyte populations. For example, human Epstein-Barr virus, a ubiquitous human DNA herpesvirus, infects more than 90% of the population worldwide. Infected lymphocytes from tonsillar tissue are a reservoir for Epstein-Barr virus infection, and these cells participate in spreading virus between individuals (3). Moreover, group C human adenoviruses (serotypes 1, 2, 5, and 6) often become latent in lymphoid tissue, especially in the tonsils (14). Both DNA replication and progeny virion production are extremely low (1 and 2%) in peripheral blood lymphocytes (16). Moreover, lymphoid organs have a central role in the pathogenesis of HIV-1 infection and can be a reservoir for the virus. Localization of HIV-1 in these organs triggers a series of events representing the normal immune response against pathogens and may contribute to the initial spreading of HIV-1.

The significance of these comparisons is that although only a low percentage of hematopoietic $CD34^+$ cells are infectable with JCV, they could be essential in disseminating virus throughout the body. Because $CD34^+$ cells are a stable population of cells, they can be a continual source of virus. In contrast, stromal cells are more susceptible to JCV infection than $CD34^+$ cells and B lymphocytes, being only about 2.5 times less susceptible than HFG cells (Table 1). Stromal cells are a major constituent of the lymphoid tissues. They are involved in lymphopoiesis in the bone marrow (12) and interact with recirculating lymphocytes. Previously, we described the interactions between human tonsillar stromal cells and B lymphocytes, and we demonstrated that B cells adhere to stromal cells through various adhesion molecules (21). Therefore, because of their function and location, we evaluated the role of these cells in polyomavirus infection. If JCV infection occurs by the respiratory route, tonsillar stromal cells, because of their relatively high susceptibility and natural interactions with lymphoid progenitors and lymphocytes, are ideally positioned to

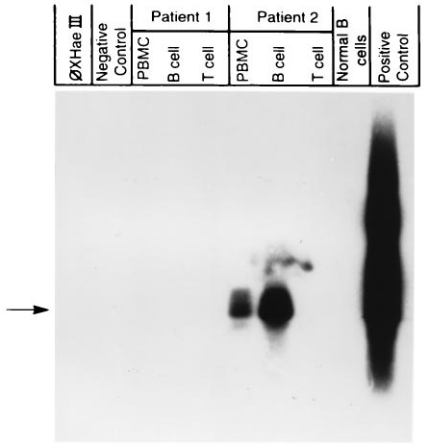

FIG. 7. Nested PCR amplification and Southern blot analysis of peripheral lymphocytes from two AIDS patients. Patient 1 did not have PML, while patient 2 was diagnosed with PML. Lanes marked patient 1 and patient 2 contain DNA extracted from unfractionated PBMC or B- and T-cell subpopulations isolated by the cell-sorting technique as shown in Fig. 6. The first lane is *Hae*III-digested ϕX DNA molecular weight markers. The negative control is PCR amplification without template. The lane marked normal B cells contains DNA extracted from the B subpopulation sorted from PBMC of a JCV-negative individual. It served as a control for virus contamination of the cell sorter. The last lane was a JCV plasmid (pM1Tc), used as a positive control.

be an initial site of infection and possibly disseminate virus. In preliminary PCR experiments, we tested tonsillar tissue from children and adult donors for JCV genomic DNA. The JCV genome was detected in one of the eight tonsils examined. We have not determined in which cell type the JCV DNA was sequestered, but these preliminary results are additional evidence that the tonsils and other lymphoid organs could be a reservoir for JCV or a site for primary infection. Another member of the human papovavirus family, BK virus (BKV), was demonstrated in tonsils of children with acute respiratory disease (13). The cell type harboring BKV DNA was not determined in that study. However, it is possible that tonsil tissue is susceptible to infection by either BKV or JCV.

The fact that cells of monocytic lineage (KG-1 and U937) were not susceptible to JCV infection in vitro supports our hypothesis of lymphoid cell susceptibility. Orenstein and Jannotta (25) demonstrated the presence of virions in the cytoplasm of phagocytes, which can be attributed to a specific internalization of cellular debris from the lytic areas. Boldorini et al. (9), however, demonstrated the presence of virions inside the nuclei of macrophages in only one of eight brain specimens from AIDS patients.

We also demonstrated that the JCV genome can be amplified by PCR from the DNA of B lymphocytes of a PML patient. These data emphasize that infection of B lymphocytes with JCV occurs in vivo. It is unclear whether the JCV found in B cells in vivo can replicate. Experiments using reverse transcription-PCR have recently shown mRNA for T protein in peripheral blood lymphocytes of PML patients (27).

Whether the presence of JCV DNA in B lymphocytes is an accurate indication of productive virus infection has not been determined. However, in support of this contention, we previously demonstrated that both HFG cells and B lymphocytes have nuclear DNA-binding proteins that interact with specific nucleotide sequences in the regulatory region of the JCV genome (22). Additionally, we recently reported that HFG cells express high level of a specific class D gene for nuclear factor 1 (29). HeLa cells, which are not permissive to JCV infection, express much lower levels of this class D gene than do HFG cells. Furthermore, our preliminary data obtained through Northern (RNA) analysis also clearly show high levels of class D gene expression by stromal cells, the cell type which is slightly less susceptible to JCV infection than HFG cells. These findings suggest that nuclear factor 1 proteins could also be important for expression of JCV in lymphoid tissue.

These studies report an expanded host range for JCV infection to include immune system cells. We are presently investigating the effects of different cytokines and chemokines on the multiplication of JCV in both progenitor cells and B lymphocytes to clarify the possible role of cell differentiation in JCV infection.

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REFERENCES

1. **Aksamit, A. J., P. Mourrain, J. L. Sever, and E. O. Major.** 1985. Progressive multifocal leukoencephalopathy: investigation of three cases using in situ hybridization with JC virus biotinylated DNA probe. Ann. Neurol. **18:**490–496.
2. **Aksamit, A. J., J. L. Sever, and E. O. Major.** 1986. Progressive multifocal leukoencephalopathy: JC virus detection by in situ hybridization compared with immunocytochemistry. Neurology **36:**499–504.
3. **Anagnostopoulos, I., M. Hummel, C. Kreschel, and H. Stein.** 1995. Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. Blood **3:**744–750.
4. **Andrews, R. G., J. W. Singer, and I. D. Bernstein.** 1990. Human hematopoietic precursors in long term culture: single CD34+ cells that lack detectable T cell, B cell and myeloid antigens produce multiple colony forming cells when cultured with marrow stromal cells. J. Exp. Med. **172:**355–358.
5. **Arthur, R. R., K. V. Shah, P. Charache, and R. Saral.** 1988. BK and JC virus infections in recipients of bone marrow transplants. J. Infect. Dis. **158:**563–569.
6. **Atwood, W. J., K. Amemiya, R. Traub, J. Harms, and E. O. Major.** 1992. Interaction of the polyomavirus, JC virus, with human B-lymphocytes. Virology **190:**716–723.
7. **Bach, J. F., and M. Dardenne.** 1972. Antigen recognition by T lymphocytes. Thymus and marrow dependence of spontaneous rosette forming cells in the mouse. Cell. Immunol. **3:**1–10.
8. **Baum, C. M., I. L. Weissman, A. S. Tsukamoto, A. M. Buckle, and B. Peault.** 1992. Isolation of a candidate human hematopoietic stem-cell population. Proc. Natl. Acad. Sci. USA **89:**2804–2808.
9. **Boldorini, R., S. Cristina, L. Vago, A. Tosoni, S. Guzzetti, and G. Costanzi.** 1993. Ultrastructural studies in the lytic phase of progressive multifocal leukoencephalopathy in AIDS patients. Ultrastruct. Pathol. **17:**599–609.
10. **Chesters, P. M., J. Heritage, and D. J. McCance.** 1983. Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. J. Infect. Dis. **147:**676–682.
11. **Dorries, K., E. Vogel, S. Gunther, and S. Czub.** 1994. Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals. Virology **198:**59–70.
12. **Dorshkind, K.** 1990. Regulation of hematopoiesis by bone marrow stromal cells and their products. Annu. Rev. Immunol. **8:**111–137.
13. **Goudsmit, J., P. Wertheim-van Dillen, A. van Strien, and J. van der Noordaa.** 1982. The role of BK virus in acute respiratory tract disease and the presence of BKV DNA in tonsils. J. Med. Virol. **10:**91–99.
14. **Green, M., W. S. M. Wold, J. K. Mackey, and P. Ridgen.** 1979. Analysis of human tonsil and cancer DNAs and RNAs for DNA sequences of group C (serotypes 1, 2, 5, and 6) human adenoviruses. Proc. Natl. Acad. Sci. USA **76:**6606–6610.
15. **Hogan, T. F., E. C. Borden, J. A. McBain, B. L. Padgett, and D. L. Walker.** 1980. Human polyomavirus infections with JC virus and BK virus in renal transplant patients. Ann. Intern. Med. **92:**373–378.
16. **Horvath, J., L. Palkonyay, and J. Weber.** 1986. Group C adenovirus DNA sequences in human lymphoid cells. J. Virol. **59:**189–192.
17. **Houff, S. A., D. Katz, C. Kufta, and E. O. Major.** 1989. A rapid method for in situ hybridization for viral DNA in brain biopsies from patients with AIDS. AIDS **3:**843–845.
18. **Houff, S. A., E. O. Major, D. A. Katz, C. V. Kufta, J. L. Sever, S. Pittaluga, J. R. Roberts, J. Gitt, N. Saini, and W. Lux.** 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.
19. **Koeffler, H. P., M. Bar-Eli, and M. C. Territo.** 1981. Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation. Cancer Res. **41:**919–926.
20. **Koeffler, H. P., R. Billing, A. J. Lusis, R. Sparkes, and D. W. Golde.** 1980. An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). Blood **56:**265–273.
21. **Lisignoli, G., M. C. G. Monaco, A. Facchini, S. Toneguzzi, L. Cattini, D. M. Hilbert, S. Lavaroni, O. Belvedere, and A. Degrassi.** 1996. In vitro cultured stromal cells from human tonsils display a distinct phenotype and induce B cell adhesion and proliferation. Eur. J. Immunol. **26:**17–27.
22. **Major, E. O., K. Amemiya, G. Elder, and S. A. Houff.** 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.
23. **Major, E. O., K. Amemiya, C. S. Tornatore, S. A. Houff, and J. R. Berger.** 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.
24. **Miller, J. S., C. M. Verfaillie, and P. B. McGlave.** 1992. The generation of natural killer cells from CD34+/DR− primitive progenitors in human long term bone marrow culture. Blood **80:**2182–2187.
25. **Orenstein, J. M., and F. Jannotta.** 1988. Human immunodeficiency virus and papovavirus infections in acquired immunodeficiency syndrome: an ultrastructural study of three cases. Hum. Pathol. **19:**350–361.
26. **Padgett, B., and D. Walker.** 1973. Prevalence of antibodies in human sera

against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. J. Infect. Dis. **127:**467–470.
27. **Pietzuch, A., M. Bodemer, S. Frye, P. Cinque, C. Trebst, W. Lueke, and T. Weber.** 1996. Expression of JCV specific cDNA in peripheral blood lymphocytes of patients with PML, HIV-1 infection, multiple sclerosis, and in healthy controls. J. Neurovirol. **2:**46.
28. **Schneider, E. M., and K. Dorries.** 1993. High frequency of polyomavirus infection in lymphoid cell preparations after allogeneic bone marrow transplantation. Transplant. Proc. **25:**1271–1273.
29. **Sumner, C., T. Shinohara, L. Durham, R. Traub, E. O. Major, and K. Amemiya.** 1996. Expression of multiple classes of the nuclear factor-1 family in the developing human brain: differential expression of two classes of NF-1 genes. J. Neurovirol. **2:**87–100.
30. **Terstappen, L. W. M. M., S. Huang, M. Safford, P. Landsdorp, and M. Loken.** 1991. Sequential generation of hematopoietic colonies derived from single non-lineage-committed CD34+CD38− progenitor cells. Blood **77:** 1218–1227.
31. **Tornatore, C. S., J. R. Berger, S. A. Houff, B. Curfman, K. Meyers, D. Winfield, and E. O. Major.** 1992. Detection of JC virus DNA in peripheral lymphocytes from patients with and without progressive multifocal leukoencephalopathy. Ann. Neurol. **31:**454–462.
32. **Verfaillie, C., K. Blakolmer, and P. McGlave.** 1990. Purified primitive human hematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated BM stroma. J. Exp. Med. **172:**509–520.
33. **Verfaillie, C. M.** 1994. Can human hematopoietic stem cells be cultured ex vivo? Stem Cells **12:**466–476.
34. **Walker, D. L., and R. J. Frisque.** 1986. The biology and molecular biology of JC virus, p. 327–377. *In* N. P. Salzman (ed.), The Papovaviridae. Plenum Press, New York.
35. **Walker, D. L., and B. L. Padgett.** 1983. Progressive multifocal leukoencephalopathy. Comp. Virol. **18:**161–193.
36. **Warren, J.** 1979. Serologic diagnosis of BKV and JCV, p. 1087–1102. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 5th ed. American Public Health Association, Inc., Washington, D.C.