Original Investigation

JC Virus in CD34+ and CD19+ Cells in Patients With Multiple Sclerosis Treated With Natalizumab

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IMPORTANCE Infection with JC virus (JCV) may lead to development of demyelinating progressive multifocal leukoencephalopathy in patients with multiple sclerosis (MS) who are treated with natalizumab.

OBJECTIVE To determine whether mononuclear cells in circulation from MS patients treated with natalizumab harbor JCV DNA.

DESIGN, SETTING, AND PARTICIPANTS In this prospective investigation, we enrolled 49 MS patients from the Clinical Center for Multiple Sclerosis at The University of Texas Southwestern Medical Center and 18 healthy volunteers. We drew 120-mL blood samples from 26 MS patients at baseline and at approximately 3-month intervals to 10 months during the course of natalizumab infusions. One blood sample was drawn from 23 MS patients receiving natalizumab for more than 24 months and from 18 healthy volunteers.

INTERVENTIONS Natalizumab treatment of MS.

MAIN OUTCOMES AND MEASURES The blood samples were separated using flow cytometry into CD34+, CD19+, and CD3+ cell subsets; DNA templates were prepared using quantitative polymerase chain reaction for JCV DNA identification. Plasma samples were tested for anti-JCV antibodies by enzyme-linked immunosorbent assays performed at the Laboratory of Molecular Medicine and Neuroscience, National Institute of Neurological and Communicative Diseases and Stroke.

RESULTS Thirteen of the 26 patients (50%) with baseline and follow-up blood samples had detectable viral DNA in at least 1 cell compartment at 1 or more points. Ten of the 23 patients (44%) receiving treatment for more than 24 months and 3 of the 18 healthy volunteers (17%) also had detectable viral DNA in 1 or more cell compartment. Fifteen of the 49 MS patients (31%) were confirmed to harbor JCV in CD34+ cells and 12 of 49 (24%) in CD19+ cells. Only 1 of 18 healthy volunteers were viremic in CD34+ cells and none in CD19+ cells. Nine patients and 1 healthy volunteer were viremic but had seronegative test results for JCV antibodies.

CONCLUSIONS AND RELEVANCE JC virus DNA was detectable within cell compartments of natalizumab-treated MS patients after treatment inception and longer. JC virus DNA may harbor in CD34+ cells in bone marrow that mobilize into the peripheral circulation at high concentrations. Latently infected cells initiate differentiation to CD19+ cells that favors growth of JCV. These data link the mechanism of natalizumab treatment with progressive multifocal leukoencephalopathy.
Since the reintroduction of natalizumab therapy in 2006 for relapsing remitting forms of multiple sclerosis (MS), more than 440 cases of progressive multifocal leukoencephalopathy (PML), the central nervous system virus-induced demyelinating disease, have been reported. The highest incidence of PML is approximately 1:80 in patients with a combination of risk factors, including receiving 24 or more natalizumab infusions, a history of immunosuppressive treatment, and seropositivity for antibodies to the JC virus (JCV). The overall incidence of PML in natalizumab-treated MS patients is 1:250, with approximately 11 new cases reported each month.1,2 Several hypotheses have focused on the direct association of natalizumab treatment and PML, particularly because few other opportunistic infections occur in this group.3 One putative mechanism centers on the lack of immune surveillance in the central nervous system because natalizumab blocks extravasation of T and B lymphocytes into the brain.4 This concept is predicated on the presence of JCV in the brain in a latent or persistent state of residency so that, at times of protracted immune suppression, a productive amplification of viral infection is established. Although this concept may be partly true, it does not explain why PML has not been reported in MS patients treated with a variety of other immunosuppressive agents (eg, azathioprine, mycophenolate, mitoxantrone hydrochloride, and rituximab). However, if protracted immune suppression was the primary mechanism of PML in such patients, we would anticipate PML occurring in MS patients whose immune systems are more severely compromised than seen in natalizumab-treated patients. Further, JCV infection in the brains of MS patients not treated with natalizumab has not been reported. The only reports of PML in MS patients are those treated with natalizumab.5

Another proposed mechanism to explain the unique link between natalizumab treatment and PML is the drug’s effect on the mobilization of mononuclear cells from the bone marrow,6,7 some of which may harbor latent JCV infection.8-10 These observations are not mutually exclusive, however, and may actually work in concert to provide the circumstances in which 2 very different demyelinating diseases can occur in the brain of a single patient.

To investigate the link between natalizumab treatment and the presence of JCV in lymphoid cells, peripheral blood samples were collected from MS patients before the first dose of natalizumab, on the day treatment was initiated, and periodically during the course of the next 10 monthly infusions. Blood samples were also collected at a single point from MS patients who received at least 24 infusions and from healthy volunteers who were matched by age and sex to the natalizumab-treated MS patients.

Methods

Patients

Twenty-six MS patients starting natalizumab therapy (Tysabri; Biogen Idec) were recruited in the Clinical Center for Multiple Sclerosis at The University of Texas Southwestern Medical Center. To isolate sufficient CD34+ and CD19+ cells, 120-mL blood samples were collected immediately before the first infusion (baseline) and then at approximately 3-month intervals, depending on the patients’ return to clinic. The samples were immediately shipped overnight to the Laboratory of Molecular Medicine and Neuroscience (LMMN), National Institute of Neurological and Communicative Diseases and Stroke, for cell separation processing. Single 120-mL samples were collected from 23 MS patients undergoing more than 24 infusions at the Clinical Center for Multiple Sclerosis, and 150-mL samples of blood were collected from the healthy volunteers at the Transfusion Medicine Blood Bank of the National Institutes of Health. Signed informed consent was obtained in accordance with the respective institutional review boards.

Cell Isolation and Phenotypic Characterization

Human CD34+ cells were enriched by positive selection using a CD34 purity selection kit (Stemcell Technologies Inc) or on lineage-depleted whole blood samples using a cocktail of monoclonal antibodies directed against CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD56, and CD66b. Peripheral blood mononuclear cells and enriched CD34+ progenitor cells were washed in culture medium (RPMI-1640; Life Technologies) without fetal bovine serum (RO medium). The cells and RO medium were mixed with pretitered amounts of directly conjugated monoclonal antibodies using the following surface stains: for CD3 and CD8, fluorescein isothiocyanate conjugate; for CD19, peridinin-chlorophyll-cyanine 5.5 conjugated reagent or brilliant violet 421; for CD4, phycoerythrin; for CD34, phycoerythrin–cyanine 7; and for CD45, V500 (all reagents, BD Biosciences) in a total volume of 100 μL of RO medium. Cells were stained for 20 minutes at 4°C, washed and resuspended in 250 μL of phosphate-buffered solution, and then passed through a 40-μm strainer to ensure single-cell suspension. Flow cytometric analysis was performed immediately after this treatment.

Flow Cytometric Analysis

Cells were analyzed using a flow cytometer (FACSAria II SORP; BD Biosciences) equipped for the detection of as many as 6 fluorescence variables. Events collected for each sample numbered from 5000 to 50 000. Electronic compensation was conducted with peripheral blood mononuclear cells stained separately with single monoclonal antibodies used in test samples. Electronic compensation was performed with antibody capture beads (BD Biosciences) for CD34+ cells only. The cells were then identified as mononuclear cells by side-scatter area and forward-scatter area and as singlet cells by forward-scatter height, forward-scatter width, side-scatter height, and side-scatter width. We defined and sorted CD34+, CD19+, and CD3+ cells by positive selection using the CD34+/CD45−, CD19+/CD45−, and CD3+/CD45− gates, respectively.

Quantitative Polymerase Chain Reaction for JCV Genome

Cell samples were examined using the validated, Clinical Laboratory Improvement Amendment–certified JCV quantitative polymerase chain reaction (PCR) method.11-13 Cellular DNA was extracted from a maximum of 5.0 × 106 cells per sample using a DNA blood and tissue kit (DNAeasy, catalogue No. 69504; Qiagen) and eluted to a final volume of 25 μL. The primers and
Probes for the specific amplification and detection of the conserved N-terminal of the JCV T protein do not amplify BK virus or simian virus 40 DNA in this region. Extracted DNA templates were amplified using the PCR reagents (TaqMan Universal reagent, catalogue No. 4304437; Applied Biosystems) and a commercially available PCR system (7500 Real-Time System; Applied Biosystems). All samples were run in duplicate using 10 μL per reaction. The number of JCV genomes was calculated using the manufacturer’s system software and adjusted according to the dilution factors before reporting as copies per milliliter only when both duplicates detected viral DNA.

**Enzyme-Linked Immunosorbent Assay for Determination of Anti-JCV Antibody**

The gene that codes for the major JCV structural protein, Vp-1, was amplified from SVG cells14 infected with the Mad-4 viral variant of persistently infected owl monkey 586 tumor cells.15 This amplified Vp1 gene was ligated into the pBlueBacIII plasmid vector and cotransfected into Sf9 insect cells along with the baculovirus DNA expression vector system (Bac-N-Blue; Invitrogen) according to the manufacturer’s protocol. Subsequent proliferating baculovirus expressing the recombinant Vp1 was used as the inoculum in Sf9 expansion cultures. Supernatant from the expansion cultures was ultracentrifuged through 40% (weight to volume ratio) sucrose cushions. The resulting recombinant Vp1 pellets were subjected to deoxycholic acid treatment, low protein-binding polyethersulfone membrane filtration, and gradient banding on density gradient medium (OptiPrep; Nycomed) to remove cell lysate contaminants. This highly purified recombinant Vp1 was used as the antigen in establishing the enzyme-linked immunosorbent assay (ELISA) for determining the level of JCV antibody response present in the clinical samples. In the ELISA, 4× serial dilutions that generated an optical density reading of 0.05 greater than that of plate and well controls were labeled as positive at a dilution of 2560. The highest dilution of a sample registering positive findings was used to identify the titer of the sample.16 We compared the LMMN ELISA assay directly with a second-generation JCV antibody ELISA (STRATIFY JCV; Focus Diagnostics) supplied as a kit by the manufacturer using 17 plasma samples from these patients. Fifteen of the 17 samples showed identical serological results, whereas 2 other samples that the LMMN ELISA showed to have seronegative results had indeterminate results using the STRATIFY JCV ELISA after step 2 adsorption with viruslike particles as described below.

**Results**

Natalizumab is known to promote mobilization of hematopoietic stem cells from the bone marrow, a putative site for JCV latency, into the peripheral circulation, resulting in higher-than-normal physiological levels of CD34+ cells for months to years after treatment. Therefore, we collected blood samples and separated them into CD34+, CD19+, and CD3+ cells using flow cytometry. The mononuclear cells were quantitated in relation to total number of peripheral blood mononuclear cells (Figure 1). We found an increased percentage of CD34+ cells from MS patients.
compared with healthy volunteers (Figure 2) as in previous observations. Percentages of CD34+ and CD19+ cells were persistently elevated in the blood samples from MS patients treated with natalizumab starting at 3 months and for extended periods.

Although all blood samples were separated into cell compartments by flow cytometry, the data in Table 1 report only those samples confirmed to harbor detectable levels of JCV DNA. Patients described in Table 1 reflect the acquisition of the samples that were sent to the LMMN for analysis and not the total number of patients in either cohort. Blood samples were collected at baseline (before infusion) and at approximately 3-month intervals to 9 or 10 months, depending on patient status, for the 26 patients initiating treatment. A total of 92 samples from this cohort (4 samples of 120 mL each) underwent analysis. Table 1 shows the study participants with viremia, the specific compartment in which viremia was detected, the number of infections, and the antibody titer of the plasma at the time of testing. Antibody titers of 2560 or greater are considered seropositive.

Table 1 also shows that 3 of 18 healthy volunteers (17%) were viremic, although only in 2 cell compartments (CD19+ cells in 2 and CD34+ cells in 1) for a mean of 66 copies/mL of viral DNA.

In the cohort just initiating treatment, 19 of 92 samples (21%) from 13 of 26 patients (50%) had detectable viral DNA. Three of these 13 patients (23%) had detectable viral DNA in CD34+ and CD19+ cells. In the cohort of 23 patients with more than 24 infusions from whom only 1 blood sample was collected, 10 (38%) had detectable viral DNA in cell compartments, including CD34+ and CD19+ cells in 1 of these patients. Regardless of the compartment in which viral DNA was detected across all 3 groups, the copy number of viral genomes was low, particularly when compared with the numbers usually seen in virus excreted into the urine, or viruria. None of the samples from any group had detectable viral DNA in the CD3+ T cells, the cell control from each patient irrespective of group designation. JC virus has not been shown to bind to or infect T cells.

When both patient groups were combined (Table 2), 15 of 49 patients (31%) were viremic in CD34+ cells at different times, including 2 patients at baseline with a mean of 25 copies/mL in the initiating group and 212 copies/mL in the longer treatment group. Twelve of the 49 patients (24%) were viremic in CD19+ cells, with a mean of 96 copies/mL in the initiating group and 543 copies/mL in the longer treatment group. The plasma viremia in these patients has been reported previously. Seventeen patients (35%) had plasma viremia. Six patients were viremic only in the plasma (patients 3, 8, 10, 33, 36, and 48), including 4 at baseline with a mean of 86 copies/mL in the initiating group and 67 copies/mL in the longer treatment group. Three patients were found to be viremic in all 3 compartments (patients 2, 13, and 40). The high copy number in cell compartments in patient 40, who had 30 infections, was much larger than those of the others. In general, however, the viral copy number was higher in the longer treatment group, with a mean of 230 copies/mL compared with 71 copies/mL in the initiating group. Analysis of viremia in the longer treatment group showed that viral DNA was primarily localized within CD34+ cells at a mean of 212 copies/mL compared with 25 copies/mL in the initiating group and within CD19+ cells at a mean of 543 copies/mL in the longer treatment group compared with 96 copies/mL in the initiating treatment group. Twenty-nine of 49 patients (59%) had viremia in 1 or more compartments.

The serological analysis revealed that 14 of 23 patients (61%) with cell-associated viremia from both groups had seronegative test results using the LMMN ELISA assay. To ensure that these results were not assay dependent, we compared the STRATIFY JCV test kit sent to the LMMN and the LMMN ELISA using the 17 plasma samples previously reported. Both assays showed very similar results. All 11 seropositive samples had the same test results in both assays. Four of the 6 seronegative samples were seronegative in both assays. Two other seronegative samples in the LMMN ELISA would be classified as indeterminate in the STRATIFY JCV assay. The observation that the seronegative individuals can be viremic in cell compartments in this study raises the possibility that lymphoid cell–associated JCV can occur in a persistent state that may not be adequate for antigen presentation and the generation of a humoral immune response.

Discussion

Natalizumab exerts highly significant attenuation of clinical and radiographic features of measures of MS disease activity. However, as a selective adhesion molecule inhibitor, natalizumab also prevents homing of hematopoietic CD34+ stem cells in the bone marrow, consequently augmenting their mobilization into the peripheral circulation. In previous reports, analysis of cell populations in the peripheral blood of MS patients at initiation and then in the first several months of natalizumab treatment showed an increase in CD34+ cells but also in the clonal frequency of circulating B cells. Levels of CD20+ and B cells increased at 6 and 12 months with reduced levels of CD49d, the α 4 integrin, and CD29, the β 3 integrin, both targeted by natalizumab. Immature B cells that can induce interleukin 10 expression are also increased. In another study, natalizumab treatment increased a selective release of bone
marrow lymphoid precursors compared with myeloid precursors, resulting in chronic increases in T- and B-cell populations in the peripheral circulation. Marginal zone–like memory B cells significantly increased expression of CD49d, which enables harboring in marginal zones in lymph nodes.

Together these studies provide evidence that supports the role of these cell populations as possible disseminators of JCV because natalizumab triggers the mobilization of cells potentially infected with JCV into the circulation. The cell populations described in the present study showed the same pattern of responses with natalizumab treatment, a finding that persisted for several years (Figure 2). The observation of JCV latency within these characterized mononuclear cells makes a direct mechanistic link between natalizumab and the occurrence of PML. We used the highly sensitive, specific, and validated quantitative PCR assay that provided the laboratory confirmation of PML diagnosis in nearly half of the MS cases in the present study to detect viral DNA in cell compartments. Detectable viral DNA in CD34+ cells was found in 15 of 49 patients (31%) and in CD19+ cells in 12 of 49 (24%) after initiation of treatment or after several years of continuous treatment.

In contrast to our findings, other studies have not identified JCV DNA in CD34+ cells, suggesting that compartments such as bone marrow or lymphoid tissues may not be reservoirs for latent infections. However, the same studies failed to identify cytomegalovirus in CD34+ cells as a positive control in their study, despite evidence from multiple reports that confirm detection of cytomegalovirus DNA25,26 and JCV DNA9,10 in these lymphoid stem cells. The assays that did not detect JCV DNA in similar cell populations probably were not sensi-

### Table 1. Detectable JCV Viremia in the Study Population*

<table>
<thead>
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<th>Study Group by Identification No.</th>
<th>No. of Infusions</th>
<th>Compartment</th>
<th>Viremia, No. of Copies/mL</th>
<th>Antibody Titer (Status)</th>
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<td>10 240 (+)</td>
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* Only viremia data for 2 cell compartments are shown. Patients 4, 12, 19, 41, and 55 were viremic in the CD34+ cell compartment and in plasma. Patients 20, 26, and 52 were viremic in the CD19+ cell compartment and in plasma. Patients 2, 13, and 40 were viremic in all 3 compartments. Viremia in plasma only was reported previously (not shown) for patients 3, 8, 10, 33, 36, and 48.
tive enough and probably were not used in sufficient numbers of cells to compare the outcomes of these investigations legitimately. In our study, more CD34+ cells were available from 120- and 150-mL blood samples that were collected and processed within 24 hours, in contrast to the 18-mL samples used in the other reported study.25 In addition, the low viral copy number in our study is consistent with a latent or a persistent infection. These observations add to the complexity of finding evidence of T-cell responses to JCV in these patients demonstrated T-cell responses to JCV proteins, indicating cell-mediated immune responses to JCV infection. These observations add to the complexity of finding a rigorous set of criteria for identifying patients at increased risk of PML when receiving natalizumab or similar therapies that modulate the immune system.

Although viral serology plays an important role in risk stratification algorithms,31 the use of serology alone may not be sufficient to identify all patients with prior exposure to JCV.32 Other measures of JCV infection may be of equal importance and should be considered, including T-cell responses, a rise in antibody titer indicating active infection, and the presence of JCV DNA variants in peripheral circulation, particularly in cell compartments.33 These additional factors, previously suggested,34 could be helpful during periodic surveillance if included in stratification protocols.35 Continued studies seem warranted on the pathoetiologic factors that influence the development of PML in patients treated with natalizumab and other biological modulators of the human immune system.

### Conclusions

We detected JCV DNA within the cell compartments of natalizumab-treated MS patients after treatment inception and after 24 months. The JCV DNA may harbor in CD34+ cells in bone marrow that mobilize into the peripheral circulation at high concentrations. Cells with latent infection initiate differentiation to CD19+ cells that favors growth of JCV. Continued studies are needed to further investigate natalizumab treatment as the mechanism of PML.

### ARTICLE INFORMATION

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**Author Contributions:** Dr Frohman had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

- **Study concept and design:** E. M. Frohman, Monaco, Remington, Jensen, Monson, T. C. Frohman, Douek, Major.
- **Acquisition, analysis, or interpretation of data:** All authors.
- **Drafting of the manuscript:** E. M. Frohman, Remington, T. C. Frohman.
- **Critical revision of the manuscript for important intellectual content:** All authors.
- **Statistical analysis:** E. M. Frohman, Johnson, Perkins.
- **Obtained funding:** E. M. Frohman, Remington, T. C. Frohman.
- **Administrative, technical, or material support:** E. M. Frohman, Monaco, Remington, Ryschkewitsch, Jensen, Monson, T. C. Frohman, Douek, Major.
- **Study supervision:** E. M. Frohman, Remington, Greenberg, T. C. Frohman, Douek, Major.

**Conflict of Interest Disclosures:** Dr Frohman has received speaking and consulting fees from Biogen.
Idec, TEVA Neuroscience, Acorda, Genzyme, and Novartis and has received consulting fees from Abbott Laboratories. Ms Remington serves on the speaker bureaus for TEVA Neurosciences and Biogen Idec and has received honoraria from TEVA Neurosciences, Biogen Idec, Genzyme, Acorda Therapeutics, the Consortium for Multiple Sclerosis Centers, and the National Multiple Sclerosis Society. Dr Greenberg has received grant support from the Accelerated Cure Project, the Guthy Jackson Charitable Foundation for NMO (Neuromyelitis Optica), and Amplimmune, Inc; has received consulting fees from Biogen Idec, sanofi-aventis, DiOGenix, The Greater Good Foundation, and Elon Pharmaceuticals; and owns equity in DiOGenix, Inc. Ms Frohman has received speaker’s and consulting fees from Biogen Idec, Novartis, Genzyme, and Acorda. No other disclosures were reported.

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REFERENCES


