Published in final edited form as:

Ann Neurol. 2014 June ; 75(6): 925–934. doi:10.1002/ana.24148.

JC virus Reactivation During Prolonged Natalizumab Monotherapy for Multiple Sclerosis

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Abstract

Objective—To determine the prevalence of JC virus (JCV) reactivation and JCV-specific cellular immune response during prolonged natalizumab treatment for multiple sclerosis (MS).

Methods—We enrolled 43 JCV-seropositive MS patients, including 32 on natalizumab monotherapy >18 months, 6 on interferon β -1a monotherapy >36 months and 5 untreated controls. We performed QPCR in cerebrospinal fluid (CSF), blood and urine for JCV DNA and we determined JCV-specific T cell responses using enzyme-linked immunosorbent spot (ELISpot) and intracellular cytokine staining (ICS) assays, *ex vivo* and after *in vitro* stimulation with JCV peptides.

Results—JCV DNA was detected in the CSF of 2/27 (7.4%) natalizumab-treated MS patients who had no symptoms or MRI lesions consistent with progressive multifocal leukoencephalopathy. JCV DNA was detected in blood of 12/43 (27.9%) and in urine of 11/43 (25.6%) subjects without difference between natalizumab-treated patients and controls. JC viral load was higher in CD34⁺ cells and in monocytes compared to other subpopulations. ICS was more sensitive than ELISpot, and JCV-specific T cell responses, mediated by both CD4⁺ and CD8⁺ T-lymphocytes, were detected more frequently after *in vitro* stimulation. JCV-specific

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Author contributions

Conceived and designed the study: IJK. Contributed to subject enrollment: EB, MCS, RPK, JAS, MD, CI, MH, GJB, IJK. Performed the experiments: SC, XD, EB, SB. Contributed reagents/materials/analysis tools: XD, IJK. Analyzed the data: SC, IJK. Wrote the paper: SC, IJK.

CD4⁺ T-cells were detected *ex vivo* more frequently in MS patients with JCV DNA in CD34⁺ (p=0.05) and B cells (p=0.03).

Interpretation—Asymptomatic JCV reactivation may occur in CSF of natalizumab-treated MS patients. JCV DNA load is higher in circulating CD34⁺ cells and monocytes compared to other mononuclear cells, and JCV in blood might trigger a JCV-specific CD4⁺ T-cell response. JCV-specific cellular immune response is highly prevalent in all JCV-seropositive MS patients, regardless of treatment.

Keywords

JC virus; natalizumab; progressive multifocal leukoencephalopathy

Introduction

As of February 2014 437 cases of progressive multifocal leukoencephalopathy (PML) have been reported in multiple sclerosis (MS) patients treated with natalizumab worldwide (<http://www.biogenidec.com>). PML is a demyelinating disease of the brain caused by the reactivation of the polyomavirus JC (JCV), usually occurring in immunosuppressed individuals with AIDS, hematologic malignancies, organ transplantation or auto-immune diseases treated with immunosuppressive medications^{1, 2}. Natalizumab is a monoclonal antibody against $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$ integrin receptors and prevents trafficking of leukocytes out of the blood stream. PML risk factors include JCV seropositivity, prior exposure to immunosuppressive agents and duration of natalizumab therapy for more than two years^{3, 4}. However, the pathogenesis of natalizumab - associated JCV reactivation is incompletely understood. Studies aimed at detecting the early events of JCV reactivation under natalizumab therapy have yielded conflicting results^{5–14}. While PML risk peaks after 2 years, most of studies spanned the first 18 months of treatment. Herein, we sought to determine the prevalence of JCV reactivation in CSF, blood and urine in MS patients after long term natalizumab use. In addition, we aimed to characterize the type of cells carrying the virus in the peripheral blood and decipher the role of cellular responses to JCV in a cross-sectional study.

Subjects and methods

Subject selection and specimen collection

This cross-sectional study was approved by our institutional review board. In order to be enrolled in the study, all MS patients had to be seropositive for JCV as determined by ELISA assay (Focus Diagnostics)¹⁵. Altogether, we collected samples from 43 MS patients, 32 of whom were on natalizumab monotherapy, 6 on interferon β -1a >36 months (range 40–62, median 52) and 5 on no treatment. Natalizumab-treated MS subjects were further divided into 3 groups based on duration of exposure to natalizumab: 14 subjects in the 18–20 months group, 7 subjects in the 22–25 months group and 11 subjects in the >35 months (range 35–73, median 41) group.

CSF, blood and urine were collected for each subject. CSF was not available for five patients on natalizumab and four on interferon β -1a. CSF was immediately sent to the

clinical laboratory for JCV DNA detection by PCR (Focus Diagnostics). Subjects and clinicians were notified immediately in the event of a positive result. Remaining CSF was stored frozen (-20°C) for batch testing. Fresh blood samples were kept at room temperature and processed within 24 hours. Peripheral blood mononuclear cells (PBMC) were separated from plasma using Ficoll gradient centrifugation. Plasma and urine were aliquoted and stored frozen (-20°C) for batch testing.

Cell sorting

Cells were sorted into six subpopulations using FACS ARIA II (Becton Dickinson). Whole blood was used for sorting polymorphonuclear cells (PMN) (forward and side scatter) and monocytes (forward and side scatter and granularity). Previously Ficoll gradient-separated mononuclear cells were used immediately for sorting other cell subpopulations, after staining with antibodies targeting the following cell-surface markers: B cells ($\text{CD19}^{+}\text{CD20}^{+}$), T cells (CD3^{+}), natural killer cells (NK; $\text{CD3}^{-}\text{CD16}^{+}\text{CD56}^{+}$), and pluripotent stem cells (CD34^{+}). Since all resulting cell subpopulations represent mononuclear cells obtained from peripheral blood, all 6 cell types will be referred to as PBMC hereafter.

DNA extraction and QPCR

Sorted PBMC fractions were suspended in 200 μL of PBS. DNA extraction was performed using the Qiagen DNA Blood Mini Kit. For CSF, plasma and urine, 200 μL of fluid was taken from the samples and used for direct extraction with the Qiagen MinElute virus Kit. We used quantitative real-time PCR (QPCR) to detect and quantify JCV DNA in all samples as previously described¹⁶. Briefly, we used primers that represent nucleotides (nt) 4298 to 4320 ($5^{\prime}\text{-AGAGTGTGGGATCCTGTGTTTT-3}^{\prime}$) and 4375 to 4352 ($5^{\prime}\text{-GAGAAGTGGGATGAAGACCTGTTT-3}^{\prime}$) of the large T gene sequence of JCV strain Mad-1. Each 25 μL reaction mixture contained 400 nM of forward and reverse primers, 100 nM of JCV-specific probe (nt 4323 to 4350) ($5^{\prime}\text{-6-carboxyfluorescein-TCATCACTGGCAAACATTTCTTCATGG C-6-carboxytetramethylrhodamine-3}^{\prime}$), (Rox reference dye and TaqMan universal PCR master mix, Applied Biosystems). To limit the possibility of PCR false positive results, all specimen manipulation took place in an isolated tissue culture room, located in a different area from our molecular laboratory. DNA extraction, storage and PCR experiments were performed separately for CSF, blood and urine so as to avoid cross-contamination. JCV-negative DNA extraction controls were performed for all specimens. In addition, no-template controls were included in all PCR runs. Experiments took place in triplicates for 40 cycles. 2 out of 3 or 3 out of 3 wells with JCV DNA detected were required for a result to be interpreted as positive, based on a standard curve that was generated in each experiment. QPCR limit of detection for CSF, plasma and urine was 140 copies/ml and 1 copy/1000 cells for PBMC subpopulations.

T cell detection by interferon- γ release assays

Interferon- γ (IFN- γ) enzyme-linked immunosorbent spot (ELISpot) and intracellular cytokine staining (ICS) assays were employed in order to detect cellular immune responses against JCV, as previously described¹⁷. Briefly, a peptide library of the major capsid protein, VP1, was used to detect JCV VP1-specific T-cells. This protein library consists of 97 15-amino-acid (aa) peptides, which overlap by 11 aa, spanning the entire VP1 protein.

The peptides are divided into four sequential pools, A to D as follows: pool A, p1 to p93 ($n=24$); pool B, p97 to p157 ($n=24$); pool C, p161 to p253 ($n=24$); and pool D, p257 to p341 ($n=25$). This overlapping peptide library allowed us to detect JCV-specific CD4⁺ or CD8⁺ T-cells regardless of the HLA alleles of the study subjects. Importantly, both assays took place using fresh blood and were repeated after stimulating PBMC with VP1 peptides and incubating with interleukin-2 (IL-2) for 11 days. Therefore, by performing these experiments both *ex vivo* and *in vitro*, we were able to evaluate the performance of ELISpot and ICS *ex vivo* for the first time simultaneously and compare *ex vivo* with *in vitro* results. Both techniques are further described in the online supplement.

For the ELISpot assay, a 96-well plate (Millipore) was prepared with diluted purified anti-human IFN- γ monoclonal antibody (Ab) (0.5 $\mu\text{g/ml}$) (B27; BD Pharmingen). After being counted with a Guava automated cell counter, 100,000 lymphocytes per well were plated in the presence of 50 μl of peptide dilution at a 2- $\mu\text{g/ml}$ final concentration. These cells were used to measure the patient's absolute response. In addition, 100,000 cells per well were stimulated with phytohemagglutinin (PHAM) (10 $\mu\text{g/ml}$) and used as positive control and 100,000 cells per well were not restimulated (either with one of the peptide pools or with PHAM) and were used to measure the baseline IFN- γ secretion. Each condition was tested in triplicate. After incubation overnight at 37°C, the cells were washed and incubated with rabbit polyclonal anti-human IFN- γ -biotin (Biosource) for 2 h at 37°C. After the plate was washed again, 100 μl of streptavidin (Southern Biotechnology; dilution of 20 μl in 10 ml ELISpot reagent buffer) was added to each well and the plate was incubated for 45 min at room temperature. The plate was then washed three times with D-PBS-Tween 20 and three times with D-PBS. Subsequently, 100 μl of nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate chromogen (Pierce) was added to each well and the plate was developed for 7 min. We then air dried the plate for 24 h before analyzing it on the ELISpot plate reader (Hitech Instruments) using Image-Pro Plus image-processing software (Media Cybernetics, Des Moines, IA). The ELISpot results were reported after subtraction of the baseline IFN- γ secretion from the patient's absolute response. A test was considered positive when the number of spot-forming units (SFU) was three times greater than the baseline, had a coefficient of variability (CV) less than 70% in triplicate wells, and was greater than 50 per 10⁶ cells after subtraction of the baseline.

For the ICS assay, 10⁶ lymphocytes, counted with the Guava automated cell counter, were suspended in 100 μl and added to 100 μl of a JCV peptide dilution, serving as the patient's absolute response. Subsequently, 10⁶ cells were added to PMA and ionomycin (1 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ respectively) to obtain a positive control and 10⁶ cells were not restimulated but also went through the ICS to obtain the baseline IFN- γ secretion. All cells were incubated for 1 h at 37°C. Subsequently, 50 μl of diluted 1% monensin (GolgiStop; BD Biosciences) were added, followed by incubation for 5 h at 37°C. The reaction was stopped at 4°C overnight. The next day cells were washed and stained with Aqua Amine dye to discern between live and dead cells (Live/Dead cell stain kit, Life technologies), washed again, then stained with surface marker antibodies for CD8 (BD Biosciences; clone SK1) and CD4 (BD Biosciences; clone L200). Subsequently, cells were fixed with 200 μl Cytotfix/Cytoperm (BD Biosciences), washed, and stained with IFN- γ -specific antibody (BD Biosciences; clone

B27) and CD3 antibody (BD Biosciences; clone SK-7). Cells were fixed with 1.5% formaldehyde-PBS before samples were analyzed with a LSRII Flow Cytometer (BD Biosciences) and FlowJo software (Tree Star), gating on lymphocytes, live cells, then CD3⁺ and then CD4⁺ and CD8⁺ cells. Approximately 10⁶ events were collected per sample. Results were expressed as % IFN- γ -producing CD4⁺ or CD8⁺ T-cells. The ICS results were reported after subtraction of the baseline IFN- γ secretion for each pool from the absolute patient's response. A test was considered positive when the percentage of IFN- γ -producing CD4⁺ or CD8⁺ T-cells was equal to or greater than twice the baseline IFN- γ secretion.

Statistical analysis

Comparisons between categorical and continuous values were performed with Fischer's exact and Wilcoxon rank sum tests, respectively. Statistical analysis was performed using Prism 5.0 software.

Results

Subject characteristics

Study subject characteristics are presented in table 1. There were no significant differences in age among study groups. All subjects had at least one PML risk factor, as JCV seropositive status was an inclusion criterion. Only 3 of 32 (9.4%) natalizumab-treated MS patients were previously exposed to immunosuppressive medications. This is comparable to the 14% estimate of prior immunosuppressive agent use in natalizumab-treated MS patients in the US³. None of the interferon-treated MS patients had prior exposure to immunosuppressive medications. Natalizumab-treated patients did not experience treatment interruptions between therapy initiation and study enrollment. None of the study subjects received corticosteroids within three months from the study time point.

JCV DNA detection by PCR in CSF, blood and urine

Results of JCV PCR in various compartments are shown in Table 2. JCV DNA was detected in the CSF of 2 out of 27 (7.4%) natalizumab-treated MS patients tested (272 and 223 copies/mL) and in none of the interferon - treated or untreated MS patients tested (n=7). No pleocytosis was found in subjects who underwent lumbar puncture. The two MS patients with detectable JCV DNA in CSF had been on natalizumab for 18 and 38 months respectively. Neither of the two patients demonstrated symptoms or radiographic findings consistent with PML or JCV-related granule cell neuronopathy (JCV GCN). The clinical laboratory reported JCV DNA in the CSF of the MS patient on the 38-month natalizumab course. Consequently, this patient underwent JCV DNA testing in CSF and brain MRI 3 and 7 months after stopping the medication. The clinical laboratory did not detect JCV DNA on repeat CSF testing and brain MRI did not reveal findings consistent with PML. Natalizumab was restarted after an 8-month interruption and additional JCV DNA testing in CSF remained negative 13 and 19 months after the initial lumbar puncture. In addition, repeat brain MRI 3, 6, 9 and 20 months after restarting the medication did not suggest PML. The patient on 18 months of natalizumab had opted to switch to another medication before CSF testing took place in our laboratory. Given the cross-sectional nature of our study, we did not repeat CSF testing.

JCV was detected in PBMC, but not in plasma, of 12 of 43 MS patients (27.9%), 9 of whom were treated with natalizumab (Table 2). JC DNA was also found in the urine of 11 MS patients (25.6%) (range 140 – 103,213 copies/ml, median 692 copies/ml) 6 of whom were on natalizumab. There was no significant difference in JCV prevalence in blood and urine, and in JC viral load in urine between natalizumab-treated patients and control subjects. Of the three natalizumab-treated patients previously on immunosuppressive agents, one was found to be viremic and another was shedding JCV in urine.

JCV DNA was found in all six PBMC subpopulations in 9 of 32 (28%) natalizumab-treated patients, and in four PBMC subpopulations in 3 of 6 (50%) interferon-treated patients (Table 3). Interestingly, the two patients with positive JCV PCR in CSF (MS11 and MS22, Table 3) did not have detectable JCV DNA in blood or urine. Taking into account the entire cohort, the number of MS patients harboring JCV DNA in each PBMC subpopulation is shown in Figure 1, ranging from 18.6% for CD34⁺ cells to 11.6% for NK cells, a difference that was not significant. We then compared JC viral load among PBMC subpopulations, which ranged from 1 to 8,335 copies/1000 cells. As shown in Figure 2, JC viral load was significantly higher in CD34⁺ cells (median = 138 copies/1000 cells) compared to B cells (median = 2 copies/1000 cells, $p=0.006$), T cells (median = 1 copy/1000 cells, $p=0.004$) and PMN (median = 4 copies/1000 cells, $p=0.002$). The viral load was also higher in monocytes (median = 54 copies/1000 cells) compared to T cells ($p=0.005$) and PMN ($p=0.004$). JC viral load remained significantly higher in CD34⁺ and monocytes, compared to other PBMC subpopulations, when only natalizumab-treated MS patients were included in this analysis. Comparison of JC viral load among natalizumab groups and between natalizumab and interferon β -1a treated patients did not yield a significant difference.

Detection of cellular immune response against JCV

JCV-specific T cells were detectable by both ELISpot and ICS, without significant differences among patients from all groups (Table 4). JCV-specific cellular response detection by ELISpot, after *in vitro* stimulation with VP1 peptides was significantly enhanced compared to *ex vivo* (36/42 tested patients versus 7/43, $p<0.001$). Similarly, cellular response detection by ICS was greater after *in vitro* stimulation compared to *ex vivo* (43/43 versus 27/43, $p<0.001$). Furthermore, ICS captured JCV-specific cellular response more frequently compared to ELISpot, both *ex vivo* ($p<0.001$) and *in vitro* ($p<0.01$). JCV-specific CD4⁺ and CD8⁺ T cells were more frequently detected by ICS in all MS patients after *in vitro* stimulation with VP1 peptides than *ex vivo* ($p<0.001$), as shown in Table 5. Finally, a JCV-specific CD4⁺ - mediated response was more frequently detected, compared to a CD8⁺ - mediated response *in vitro* ($p=0.05$) (table 5).

Correlation between the cellular immune response and JC viremia

The presence of JCV-specific CD4⁺ T cells in the peripheral blood, as detected by ICS *ex vivo*, correlated with DNA detection in PBMC of natalizumab-treated MS patients ($p=0.05$). When all MS patients were included and the same analysis was repeated, we observed an association between JCV-specific CD4⁺ T cell response and detection of JCV DNA in CD34⁺ and in B cells ($p=0.05$ and $p=0.03$ respectively). We did not find an association between T cell response to JCV and presence of JCV DNA in CSF or urine.

Discussion

This study allowed us to uncover that asymptomatic JCV reactivation may occur in the CSF of natalizumab-treated MS patients. Presence of the virus in CSF is typically concurrent with PML¹⁸ or JCV-GCN¹⁹. Neither of the two patients with detectable JCV in CSF experienced symptoms or MRI findings consistent with PML or JCV-GCN. JCV DNA can be detected in rare instances in CSF of MS patients not on natalizumab^{20, 21} and of HIV-infected patients without PML²². Sadiq et al has reported JCV DNA in CSF of 2 of 200 natalizumab-treated MS patients without evidence of PML¹³. The prevalence of JCV in CSF tended to be higher in our study compared to the latter study (7.4% versus 1%, $p=0.07$). This could be explained by several differences. In our cohort, all patients were JCV seropositive, whereas Sadiq et al did not address antibody status. Furthermore, Sadiq et al relied entirely on commercial PCR with a reported limit of detection of 500 copies/ml. Although the primers and probes of commercial PCR assays remain proprietary, sensitivity of the PCR technique is crucial for JCV detection. Indeed, both CSF PCR positive patients had low JC viral load in our study, and clinical laboratory detected JCV in CSF of only one patient. In addition, the two cohorts differ substantially in duration of natalizumab treatment: at least 18 months in our cohort, versus up to 18 months in the study by Sadiq et al²³. Even taking into account differences in PCR sensitivity, these findings suggest that intrathecal JCV reactivation may depend, at least in part, on the duration of natalizumab exposure in MS patients.

Our assessment of asymptomatic JCV detection in CSF of natalizumab-treated MS patients relied on the absence of clinical or MRI features of PML. MRI is considered to be a sensitive diagnostic tool in natalizumab-associated PML^{23, 24} and has allowed early PML diagnosis, even in cases with subtle or absent symptomatology^{25, 26}. The spectrum of MRI findings is broad. Unlike PML in HIV-infected individuals, small punctuate contrast-enhancing lesions suggestive of perivascular inflammation may be present early in natalizumab-associated cases^{23–26}. Nonetheless, the underlying mechanism for these MRI features is incompletely understood as correlation with histopathology, concomitant to MRI testing, is not available in most cases. Recently, we examined the whole brain of a natalizumab-treated MS patient who died from PML, where the latest MRI was performed 12 days prior to postmortem examination²⁷. All PML lesions seen on MRI were large and easily identified histologically. JCV-infected oligodendrocytes, demyelination and T cell infiltration were found in PML lesions. However, it is possible that early nidus of JCV infection in a few brain cells or microscopic PML lesions may not be evident on MRI.

Subclinical JCV reactivation may also occur in blood of natalizumab-treated MS patients, as we and others have previously reported^{6–8, 13, 28}. We found JCV in blood of 9 of 32 (28.1%) natalizumab-treated patients, a result comparable to viremia of 35% in a different cohort, recently reported by Major et al²⁹. These results differ from large studies reported by Rudick et al³⁰ where JCV DNA was found only sporadically in blood from natalizumab-treated patients. However, this can be explained by technical aspects of collecting samples from multiple sites compared to single center studies, as discussed by Major et al³¹. In the present study, JCV DNA detection took place separately within six purified subpopulations of circulating mononuclear cells. This represents a novel approach, as opposed to detecting

JCV in unsorted PBMC simultaneously^{6, 7, 10} and may explain why we also found JCV DNA in cells of interferon-treated patients.

This methodology enabled us to make interesting observations. First, JCV is associated with multiple circulating mononuclear cell subpopulations. We and others reported similar nonspecific JCV association with leukocyte subpopulations in HIV-infected individuals with and without PML^{22, 32}. Second, in natalizumab-treated MS patients, a higher JC viral load is detected in circulating progenitor CD34⁺ cells and monocytes, compared to other circulating mononuclear cell subpopulations. The ability of JCV to infect CD34⁺ cells and B lymphocytes, has clearly been demonstrated in vitro^{33–35}, but to date JCV has not been found in CD34⁺ cells from clinical specimens of HIV-infected or natalizumab-treated patients^{36–38}. In recent studies, Saure et al³⁷ and Warnke et al³⁸ did not detect JCV in CD34⁺ cells obtained from bone marrow or blood, respectively, of natalizumab-treated patients. However, in the former study³⁷ JCV PCR was performed in only 9 MS natalizumab-treated patients; in the latter study³⁸ the average number of natalizumab infusions was approximately twelve.

The finding of high viral load in CD34⁺ cells may be in line with the hypothesis that in natalizumab-treated patients the virus enters the circulation from hematopoietic latency sites^{2, 39}. In this scenario, natalizumab hampers bone marrow homing of JCV-harboring CD34⁺ and B cells and these cells are thought to be appropriate for viral proliferation^{34, 39}. However, although we detected substantial viral load in CD34⁺ cells and monocytes compared to other cell subpopulations, this was not the case with B cells. In addition, whether JCV actively replicates within those cell subpopulations could not be answered by our study, since we did not test for viral RNA production. The site of JCV replication in blood remains unclear, since JCV RNA has very rarely been detected in blood of HIV-infected patients and not in interferon-treated or untreated MS patients^{5, 40, 41}. Furthermore, since natalizumab prevents cell diapedesis, it is not known how JCV may enter the central nervous system, even if it is carried by certain mononuclear cells in blood. In fact, it has been shown that natalizumab decreases the migratory capacity of CD34⁺ cells³⁷, monocytes and other leukocytes⁴² that possess the $\alpha 4$ integrin receptor. In addition, we did not detect JCV DNA in plasma and therefore it remained elusive whether JCV may enter the brain as cell-free virus. Consistent with previous studies, we did not find any association between viruria and viremia^{6, 7, 30}.

Given all the above, reactivation of latent JCV from within the central nervous system remains a plausible scenario in the pathogenesis of PML. Previous studies of post mortem samples have documented JCV DNA detection in brain tissue, from individuals without PML^{43–47}. Therefore, in this setting, it has been suggested that JCV DNA detection in brain tissue is consistent with latent infection. The cause of viral reactivation in brain cells, or in extraneural sites, is not known. Nonetheless, we hypothesize that JCV reactivation might be due to reduced local immuno-surveillance caused by natalizumab treatment^{42, 48}. Therefore, during treatment with natalizumab, PML could potentially develop due to reactivation from latent brain infection. In our study, natalizumab-treated MS patients with detectable JCV DNA in CSF did not have pleocytosis and were not viremic. Therefore, we believe that presence of cell-free JCV DNA in CSF indicates viral activity within the CNS.

For the first time, in the current study, we measured JCV-specific cellular response in MS patients both *ex vivo* and after *in vitro* stimulation with peptides spanning the entire viral capsid protein VP1, using two different techniques, ELISpot and ICS. Given the small absolute number of JCV-specific circulating T cells, their detection usually relies on stimulation *in vitro* with viral peptides. This elaborate effort led us to interesting findings. First, using both techniques, detection of JCV-specific T cells was markedly enhanced after *in vitro* stimulation. Second, we found no significant differences when comparing cellular responses against the virus among natalizumab, interferon β -1a and untreated patient groups. Third, a CD4⁺ T cell mediated JCV-specific response, detectable *ex vivo*, may be associated with presence of JCV in circulating mononuclear cells. Conceivably, viremia could trigger a virus-specific immune response. A similar correlation between JCV detection in CSF or urine and JCV-specific T-cell response was not found. Furthermore, in all MS patients, regardless of treatment, a detectable JCV-specific response in blood was more frequently mediated by CD4⁺ than CD8⁺ T-cells after *in vitro* stimulation with JCV peptides, which is not what we observe in HIV-infected PML patients and healthy individuals, where the CD4⁺ and CD8⁺ T-cell responses are detected in similar frequency¹⁷.

Since none of our natalizumab-treated MS patients developed PML, including patients with JCV in CSF or blood, the above findings suggest that a robust Th1 immune response, mediated by interferon γ – producing CD4⁺ T cells, may be key in controlling progression to PML in this patient population. This is in agreement with recent data published by Perkins et al¹² reporting a completely absent or aberrant, Th2 anti-inflammatory, cellular immune response against JCV in natalizumab-associated PML cases. It is therefore possible that the presence of Th2 response may play a role in PML development or progression, whereas Th1 cellular response against the virus may be of paramount importance in controlling clinically evident disease. Additional supporting data is available in longitudinal studies, where Hendel-Chavez et al⁹ and Jilek et al¹⁰ reported highly prevalent anti-JCV memory and effector T cell response in natalizumab-treated MS patients. Finally, Aly et al⁴⁹ observed a central role of anti-VP1 CD4⁺ T cells in PML lesions of MS patients with immune reconstitution inflammatory syndrome following natalizumab discontinuation. Taken together, these findings suggest that a vigorous JCV-specific Th1 cellular immune response may be protective against natalizumab-associated PML.

Our study has limitations. The size of our cohort is small and the cross-sectional nature of our study did not allow for longitudinal follow-up. However, PML remains a rare complication of treatment with natalizumab and JCV detection in CSF requires treatment interruption, given the potential risk for PML. Both of the above limit the design of longitudinal studies which would aim to investigate the sequence of events in natalizumab-associated JCV reactivation and PML.

Our findings contribute to the understanding of JCV reactivation during treatment with natalizumab. Our results show that: after natalizumab treatment for at least 18 months, high JC viral load may be found in circulating CD34⁺ and monocytes; viremia may be associated with a JCV-specific CD4⁺ T cell - mediated response; and the virus might reactivate in CSF subclinically. In addition, our findings suggest that JCV detection in peripheral blood mononuclear cells, but not in plasma or urine, may be a surrogate marker for viral

reactivation in natalizumab-treated MS patients. However, whether viremia precedes intrathecal reactivation and whether subclinical reactivation in CSF is essentially a prelude to PML are questions which remain unsettled. In conclusion, PCR detection of JCV DNA in CSF after extended natalizumab course, in absence of new clinical or MRI findings, is not equivalent to PML. A combined clinical and radiographic assessment of natalizumab-treated MS patients remains key in monitoring for PML and should guide testing for JCV in CSF. As the number of natalizumab-associated PML cases continues to increase, understanding the pathogenesis of JCV reactivation and improving PML risk mitigation strategies are urgent issues in the care of MS patients on natalizumab.

Acknowledgement statement

This study was supported by grants from the National Multiple Sclerosis Society RG 4523-A-1 and Biogen Idec to I.J.K. I.J.K. is also supported in part by NIH grants R01 NS 047029, R01 NS 074995, and K24 NS 060950. We thank Sarah Gheuens and Elizabeth Norton for helpful discussions and technical assistance in optimizing and performing the immunologic assays.

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Figure 1.

Frequency of JCV DNA detection by PCR in PBMC subpopulations and plasma of all MS patients. No significant differences were found between natalizumab and interferon β -1a treated patients.

Figure 2.

Measurement of JC viral load in blood mononuclear cell subpopulations of all MS patients. JC viral load was higher in CD34⁺ cells compared to B cells (p=0.006), T cells (p=0.004) and polymorphonuclear (PMN) cells (p=0.002). Viral load was also higher in monocytes (Mono) compared to T cells (p=0.005) and PMN (p=0.004). JC viral load was significantly higher in CD34⁺ and monocytes, compared to other PBMC subpopulations, when only natalizumab-treated MS patients were included in this analysis.

Table 1

Characteristics of MS patients

	NTZ 18–20 mo	NTZ 22–25 mo	NTZ >35 mo	IFN β -1a > 36mo	Untreated
Total # of patients	14	7	11	6	5
Age range (median)	(28–72) 45	(31–64) 46	(23–58) 50	(42–57) 45	(30–62) 33
No. males:females 14 : 29	7 : 7	0 : 7	4 : 7	2 : 4	1 : 4
Number of patients previously on immunosuppressive agents *	3	0	0	0	0

* mitoxantrone, cyclophosphamide, azathioprine

Table 2

JCV DNA detection by QPCR in CSF, blood and urine of MS patients

	NTZ 18–20 mo	NTZ 22–25 mo	NTZ >35 mo	IFN β -1a > 36mo	Untreated
CSF* 2/34 (5.9%)	1/11	0/6	1/10	0/2	0/5
Plasma	0/14	0/7	0/11	0/6	0/5
PBMC 12/43 (27.9%)	4/14	2/7	3/11	3/6	0/5
Urine 11/43 (25.6%)	3/14	1/7	2/11	4/6	1/5

* No CSF result was available for 5 natalizumab-treated patients and 4 interferon-treated patients. Patients with detectable JCV DNA in CSF did not demonstrate symptoms or radiographic findings consistent with PML or JCV granule cell neuronopathy.

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Table 3

JCV DNA in PBMC subpopulations, CSF and urine of MS patients

	CD34	B	Mono	T	NK	PMN	CSF	Urine
NTZ 18–20 mo								
MS04*	+	+	+	+	+	+		
MS17**								+
MS19	+		+	+	+			
MS22							+	
MS25	+	+						+
MS33	+		+	+		+	n/a	
MS34								+
NTZ 22–25 mo								
MS12				+				
MS29		+						
MS38								+
NTZ >35 mo								
MS02	+	+	+	+	+	+	n/a	
MS03	+	+	+	+	+	+		+
MS11							+	
MS18								+
MS27	+	+	+		+	+		
IFN β -1a >36 mo								
MS05	+	+						+
MS40							n/a	+
MS41							n/a	+
MS42							n/a	+
MS43						+	n/a	
Untreated								
MS23								+

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previously on azathioprine
*
**
previously on mitoxantrone

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Table 4

ICV-specific cellular immune response detected by ELISpot and ICS

	NTZ 18–20 mo	NTZ 22–25 mo	NTZ >35 mo	IFN β -1a> 36 mo	Untreated	All subjects
<i>ex vivo</i>	3/14 (21.4%)	1/7 (14.3%)	0/11 (0%)	2/6 (33%)	1/5 (20%)	7/43 (16.3%)
ELISpot						p<0.001
<i>in vitro</i>	11/14 (78.6%)	7/7 (100%)	10/10 (100%)	3/6 (50%)	5/5 (100%)	36/42 (85.7%)
<i>ex vivo</i>	8/14 (57.1%)	5/7 (71.4%)	9/11 (81.9%)	3/6 (50%)	2/5 (40%)	27/43 (62.8%)
ICS						p<0.001
<i>in vitro</i>	14/14 (100%)	7/7 (100%)	11/11 (100%)	6/6 (100%)	5/5 (100%)	43/43 (100%)

Table 5

JCV-specific CD4⁺ and CD8⁺ T cell responses detected by ICS

	NTZ 18–20 mo	NTZ 22–25 mo	NTZ >35mo	IFN β -1a> 36mo	Untreated	All subjects
<i>ex vivo</i>	7/14 (50%)	4/7 (57.1%)	8/11 (72.7%)	2/6 (33%)	2/5 (40%)	23/43 (53.4%)
CD4⁺						p<0.001
<i>in vitro</i>	14/14 (100%)	7/7 (100%)	11/11 (100%)	6/6 (100%)	5/5 (100%)	43/43 (100%)
<i>ex vivo</i>	5/14 (35.7%)	2/7 (28.6)	8/11 (72.7%)	3/6 (50%)	2/5 (40%)	20/43 (46.5%)
CD8⁺						p<0.001
<i>in vitro</i>	12/14 (85.7%)	6/7 (85.7%)	11/11 (100%)	6/6 (100%)	3/5 (60%)	38/43 (88.4%)