Journal of NeuroVirology, 15: 351–359, 2009

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.3109/13550280903131923

informa
healthcare

Reactivation of BK polyomavirus in patients with multiple sclerosis receiving natalizumab therapy

Roisin M Lonergan,[1] Michael J Carr,[2] Cillian F De Gascun,[2] Lisa F Costelloe,[1] Allison Waters,[2] Suzie Coughlan,[2] Marguerite Duggan,[1] Katie Doyle,[1] Sinead Jordan,[1] Michael W Hutchinson,[1] William W Hall,[2] and Niall J Tubridy[1]

[1]Department of Neurology, St Vincent's University Hospital, Elm Park, Dublin, Ireland; and [2]National Virus Reference Laboratory, University College Dublin, Belfield, Dublin, Ireland

Natalizumab therapy in multiple sclerosis has been associated with JC polyomavirus–induced progressive multifocal leucoencephalopathy. We hypothesized that natalizumab may also lead to reactivation of BK, a related human polyomavirus capable of causing morbidity in immunosuppressed groups. Patients with relapsing remitting multiple sclerosis treated with natalizumab were prospectively monitored for reactivation of BK virus in blood and urine samples, and for evidence of associated renal dysfunction. In this cohort, JC and BK DNA in blood and urine; cytomegalovirus (CMV) DNA in blood and urine; CD4 and CD8 T-lymphocyte counts and ratios in peripheral blood; and renal function were monitored at regular intervals. BK subtyping and noncoding control region sequencing was performed on samples demonstrating reactivation. Prior to commencement of natalizumab therapy, 3 of 36 patients with multiple sclerosis (8.3%) had BK viruria and BK reactivation occurred in 12 of 54 patients (22.2%). BK viruria was transient in 7, continuous in 2 patients, and persistent viruria was associated with transient viremia. Concomitant JC and CMV viral loads were undetectable. CD4:CD8 ratios fluctuated, but absolute CD4 counts did not fall below normal limits. In four of seven patients with BK virus reactivation, transient reductions in CD4 counts were observed at onset of BK viruria: these resolved in three of four patients on resuppression of BK replication. No renal dysfunction was observed in the cohort. BK virus reactivation can occur during natalizumab therapy; however, the significance in the absence of renal dysfunction is unclear. We propose regular monitoring for BK reactivation or at least for evidence of renal dysfunction in patients receiving natalizumab. *Journal of NeuroVirology* (2009) **15**, 351–359.

Keywords: BK virus; multiple sclerosis; natalizumab; reactivation

Introduction

Natalizumab (Tysabri; Biogen Idec and Elan Pharmaceuticals) is an α4-integrin monoclonal antibody that reduces the migration of leukocytes into sites of inflammation within the central nervous system (CNS) in patients with multiple sclerosis (MS). In patients with active relapsing remitting disease (RRMS), natalizumab significantly decreases the annual relapse rate and the rate of disability when compared with placebo (Kappos *et al*, 2007).

Concerns regarding the impact of natalizumab on the immune system arose when progressive multifocal leucoencephalopathy (PML) was reported in two patients with RRMS who had received the drug in combination with Avonex (interferon-beta 1a), and in one patient who received natalizumab alone as treatment for Crohn's disease (Khalili *et al*, 2007; Yousry *et al*, 2006). PML is a demyelinating disorder of the central nervous system (CNS)

Address correspondence to Dr. Roisin Lonergan, Department of Neurology, St. Vincent's University Hospital, Dublin 4, Ireland. E-mail: roisin.lonergan@st-vincents.ie; or to Professor William W. Hall, National Virus Reference Laboratory, University College Dublin, Belfield, Dublin 4, Ireland. E-mail: william.hall@ucd.ie

Roisin M. Lonergan, Michael J. Carr, and Cillian F. De Gascun contributed equally to this study.

Received 14 January 2009; revised 6 April 2009; accepted 25 May 2009.

caused by reactivation of latent JC polyomavirus (JCV) infection, typically seen in severely immunocompromised individuals. More recently, four further cases of PML associated with natalizumab use in MS have been publicized (FDA, 2008). Nevertheless, during the timeframe of the clinical trials for MS, natalizumab caused no increases in the incidence of other opportunistic infections. However, the drug was subsequently shown to reduce the CD4:CD8 ratio in the cerebrospinal fluid (CSF) of patients with MS to a degree comparable to that found in infection with the human immunodeficiency virus (HIV) (Stuve *et al*, 2006).

Polyomavirus hominis 1 (BK polyomavirus) was first isolated from the urine of a renal transplant recipient in 1971 (Gardner *et al*, 1971). Subsequent population studies demonstrated BK exposure to be widespread, with a peak seroprevalence of 60% to 100% in early childhood (Knowles, 2002). Primary BKV infection is generally asymptomatic, although fever and upper respiratory tract symptoms have been reported. As with other polyomaviruses, BK has the capacity to remain latent for life following primary infection, and BK is thought to reside predominantly in the renal tract (Reploeg *et al*, 2001). In states of relative or absolute cellular immunodeficiency, such as those found in renal transplant (RT) or hematopoietic stem cell transplant (HSCT) recipients, the virus can become reactivated and cause disease, typically affecting the kidney, and manifesting as haemorrhagic cystitis (Arthur *et al*, 1986), ureteric stenosis or polyomavirus-associated nephropathy (PAN) (Rosen *et al*, 1983). However, pulmonary (pneumonitis) and neurological syndromes (meningoencephalitis, retinitis) have been reported (Reploeg *et al*, 2001). BK reactivation (as evidenced by viruria) is also more common in HIV-infected patients than in noninfected controls (Behzad-Behbahani *et al*, 2004).

BK virus (BKV) reactivation has been described in nonimmunocompromised populations (Rodrigues *et al*, 2007; Zhong *et al*, 2007). Although the prevalence of BK viruria in some studies was not associated with mildly immunosuppressive illnesses/states such as diabetes mellitus or pregnancy, others have reported a significant degree of BK reactivation (34.6%) in pregnancy (Kalvatchev *et al*, 2008). The temporal pattern of BK reactivation in healthy individuals appears to be intermittent rather than continuous (Behzad-Behbahani *et al*, 2004; Polo *et al*, 2004). There is no known association between BKV and MS.

As six cases of PML have been reported in patients with MS receiving natalizumab, this raises the possibility that either this group as a whole is more immunocompromised than previously suspected, or that natalizumab has a more profound effect on the immune system than was previously believed to be the case. To date, the extent of viral pathology as a direct result of compromised immunity— whatever the cause—in this patient cohort is unknown, although reactivation of human herpesvirus 6 (HHV6) has been demonstrated (Yao *et al*, 2008). Thus, we present here what we believe to be the first report of BKV reactivation in this group.

Results

Fifty-seven patients, 41 female (72%), 16 male (28%), have been enrolled to date. The mean age was 36.1 years (range 17.1 to 57.2 years) and mean duration of RRMS was 6.4 years (range 2 months to 25 years). The mean EDSS at baseline was 3.1 (median 3; range 0 to 6.5) and mean duration of previous IMT was 44 months (range 0 to 180 months). Seven patients were IMT naïve. Thirty-one patients had received a single agent previously; 14 had been exposed to two agents; and 5 exposed to three agents. A mean of 15 doses (range 2 to 29) of natalizumab was received by each patient, providing a total follow-up period of ~65 patient years. Six patients have discontinued therapy: three due to progressive disability; two due to a hypersensitivity reaction (after four and seven doses, respectively); and one due to a revised diagnosis of neuromyelitis optica (Table 1).

BK viruria was detected pretreatment in 3 of 36 (8.3%) patients for whom pretreatment samples were available. BK viruria cleared within 1 month in these three patients who were positive pretreatment. In two of these patients, BK was detected in urine again within 4 months. However, these three patients were not included in the BKV reactivation cohort (described below) as they were positive at baseline. BK was not detected in baseline samples for the remaining 21 patients who had already commenced treatment. BK viremia was not detected in any of the patients at time of enrollment. Of note, JC viruria was detected pretreatment in 5 of 36 (13.9%) patients. JC viremia occurred in one patient after a single dose, and clearance occurred 7 months later, after dose 8. No patient was found to have concomitant reactivation of JC and BK viruses (urine).

BKV reactivation occurred in 12 (7 female [58%], 5 male [42%]) of 54 patients (22.2%). Characteristics for this group are presented in Table 2. Mean age was 39.2 (range 21.8 to 57.2); mean EDSS was 3.3 (range 1.5 to 5.5); mean duration of RRMS was 7.3 years (range 1 to 19); and prior IMT had been discontinued by an average of 12.2 (range 4 to 52) weeks prior to treatment. BK viruria occurred in 12 patients after a mean of 11.2 doses (range 1 to 23) and cleared in 7 patients within a mean of 7.6 months (range 1 to 10) of reactivation, and persists in 2 patients after 26 and 29 doses respectively; data are outstanding for 3 patients who discontinued natalizumab. Mean urine load at reactivation was 278632 copies/ml (range: <100 to 2964699). Two of 12 patients developed BK viremia after 7 and 8 doses, with viral loads of <100 and 17397 copies/ml, respectively.

Table 1 Clinical characteristics of 57 MS patients on natalizumab evaluated for BK reactivation

Factor	BK reactivation (n = 12)	No BK reactivation (n = 45)
Age		
Mean (range)	39.2 yrs (21.8–57.2 yrs)	32.7 yrs (17.1–57.2 yrs)
Median	25.2 yrs	35.4 yrs
Ethnicity	Irish	Irish
Gender		
Male	5	11
Female	7	34
Duration of MS		
Mean (range)	7.3 yrs (1–19 yrs)	6.22 yrs (2 months–25 yrs)
Median	5 yrs	4 yrs
EDSS		
Mean (range)	3.3 (1.5–5.5)	3.02 (0–6.5)
Median	3.0	3.0
Previous IMT*		
Interferon β	10	38
Glatiramer acetate	2	14
Mitoxantrone	0	2
IMT naive	2	5
Number of doses of natalizumab		
Mean	20.58	13.38
Median	26	12
Mean absolute CD4 count at enrollment (10^6/L)	1379.75	907.62
Mean absolute CD8 count at enrollment (10^6/L)	867.92	488.03
Mean CD4:CD8 ratio at enrollment	2.6	2.23
Mean absolute CD4 count at BK reactivation (10^6/L)	1329.42	–
Mean absolute CD8 count at BK reactivation (10^6/L)	795.67	–
Mean CD4:CD8 ratio at BK reactivation	1.9	–

*IMT = immunmodulatory therapy.

Viremia cleared within 1 month in one patient, and within 10 months in the second: the former patient remains BK viruric. No evidence for concurrent cytomegalovirus (CMV) reactivation was observed in those patients with reactivating BK virus.

CD4 and CD8 counts and CD4:CD8 ratios fluctuated within the normal range over the treatment period. There were small, transient decreases in absolute CD4 counts preceding BKV reactivation in four of seven patients, the resolution of which coincided with viral clearance in three of four cases. Renal profiles remained within normal limits in all patients: sodium (normal range 135 to 145 mmol/L), potassium (normal range 3.5 to 5.0 mmol/L), chloride (normal range 95 to 108 mmol/L), urea (normal range 2.1 to 7.1 mmol/L), and creatinine (44 to 80 mmol/L).

A 327-bp fragment of the BKV VP1 gene was amplified by nested polymerase chain reaction (PCR) directly from urine specimens of 8 of the 12 patients reactivating BKV in our cohort. Figure 1 shows a maximum likelihood phylogenetic tree of eight BKV VP1 sequences with reference sequences from GenBank for the four main BKV subtypes (I to IV) and subtype I viruses are further subdivided into clades Ia, Ib-1, Ib-2, and Ic. No discrete genetic clade was apparent for the patients in our study in whom BKV reactivated and a substantial heterogeneity in genotype of reactivating BK virus was observed. Six of the eight patients reactivated BKV subtype I virus: three patients (002T, 017T, and 026T) segregated with the predominantly European clade Ib-2 (Ikegawa *et al*, 2006; Zhong *et al*, 2009); two (patients 007T; 012T) with the predominantly African BKV clade Ia (Ikegawa et al., 2006); and a single case (016T) with the Ib-1 subtype found in significant numbers in southeast Asia (Ikegawa *et al*, 2006, Nishimoto *et al*, 2007; Zhong *et al*, 2009). Single cases were seen of the second most prevalent BK subtype, IV (patient 011T), found in both Europe and Asia (Nishimoto *et al*, 2007; Zhong *et al*, 2009), and finally, the rare BK virus subtype II was identified in the patient (006T), who shed the virus in his urine for the longest period of time in our cohort and who was also transiently viremic.

In order to determine whether genetic rearrangement (duplications, deletions, or insertions) had occurred in the noncoding control region (NCCR) of this patient (006T), who was viruric for the longest period of the study, urine samples 7 months apart were retrospectively analyzed to determine the NCCR organisation. No evidence for genetic rearrangement was observed and the two samples were found to be 100% identical over the BKV NCCR P-Q-R-S promoter/enhancer transcriptional elements with a functional initiation codon for the downstream agnoprotein gene.

Discussion

BK reactivation occurred in 12 of 54 (22.2%) patients who had received a mean of eight doses of natalizumab. This compares with a baseline prevalence of 3 in 36 (8.3%) in those patients who provided urine samples prior to commencing natalizumab. These data suggest that, in this patient group, MS, either alone or in combination with another immunomodulatory therapy (IMT), is not sufficient to account for the high rate of BK reactivation observed. However, the numbers analysed are small. Furthermore, as the rate of BK reactivation varies widely in nonimmunocompromised populations (Behzad-Behbahani *et al*, 2004; Rodrigues *et al*, 2007; Zhong *et al*, 2007), similar variability may be expected in patients with MS.

The degree of immunosuppression required for BKV reactivation is not known. Some studies performed in HIV infection have reported a correlation between BKV viruria and degree of immunodeficiency as measured by CD4 count (Behzad-Behbahani *et al*, 2004; Sundsfjord *et al*, 1994), whereas others have failed to demonstrate such a correlation

Table 2 Characteristics of patients with BK reactivation

Patient code	Sex	EDSS at baseline	Dose at which BK reactivation occurred (viruria unless indicated)	Dose at which BK was cleared (viruria unless indicated)	CD4 count×10^6/L at baseline [ref. Range 380–1500]	CD4 count×10^6/L on reactivation of BK	CD4 count×10^6/L On clearance of BK	Most recent CD4 count×10^6/L	BK virus load on reactivation, copies per ml (log_{10} copies)
002T	F	5.5	11	18	1368	1329	1856	1605	1430 (3.16)
006T	M	2.5	7*	8*	1233	1080	N/A	1305	14016 (4.15)
007T	M	5.0	8*	18 (D/C)	925	822	1026	N/A	10877 (4.04)
009T	F	2.5	15	D/C	1540	1882	N/A	N/A	<100 (<2)
011T	F	5.0	13	19	1784	1893	1807	1769	293940 (5.47)
012T	F	1.5	10	13	2489	1959	2467	1231	26173 (4.42)
014T	F	2.5	6	D/C	1270	1084	N/A	768	<100 (<2)
015T	F	4.5	17	26	1095	822	816	1302	23523 (4.37)
016T	M	3.0	23	27	840	894	918	985	546 (2.74)
017T	F	2.0	22	32	2102	1999	N/A	2374	2964699 (6.47)
026T	M	3.0	1	4	770	1634	1474	1529	2124 (3.33)
041T	M	2.5	1	D/C	1141	1311	N/A	N/A	6257 (3.80)

*Patients in whom viremia also occurred; patient 006T remains viruric.
D/C: discontinued; N/A: not available.

(Markowitz *et al*, 1993). The pattern of BK reactivation has also not been well described. One study reported that 89% of those patients excreting BKV in urine do so intermittently (Behzad-Behbahani *et al*, 2004). In a large Portuguese study ($n=498$) looking at the prevalence of polyomavirus excretion in the urine of healthy individuals, an excretion rate of BK virus of 1.8% was observed (Rodrigues *et al*, 2007). Also BK shedding in urine for individuals in the 26- to 40-year age bracket was of the same order to other age groups (1.7%), which is comparable to the average age of patients in the present study. In contrast, a study looking at polyomavirus shedding in immunocompetent individuals in Spain found a rate of urinary excretion for BK virus of ~15% (Polo *et al*, 2004). Furthermore, this Spanish study described a number of temporal patterns of polyomavirus excretion in healthy individuals, namely occasional excreters, short-term, long-term, and continuous excreters. Importantly, in the 28 patients followed, no healthy person exhibited a continuous pattern of BK virus excretion over the study period. This is in marked contrast to our two patients, who were negative at baseline for BK virus and then continuously positive post drug treatment. No Irish BK prevalence data exist in a healthy population in order to determine a relevant baseline. However, a recent large study by Egli and colleagues (2009) of 400 healthy blood donors showed a level of BK viruria of 7%, which is comparable to our findings for MS patients not receiving natalizumab and intermediate to the data presented previously for Spain and Portugal. Furthermore, Schaffer and colleagues (2006) have determined a prevalence for excretion of JCV of 20% in a cohort of healthy Irish controls, which is comparable to the recent Swiss data of 19%, suggesting this comparison may well be meaningful.

The immune response to BKV has been studied in at risk populations, though not in MS. Humoral and cell-mediated responses have been associated with the control of BK replication (Wong *et al*, 2007), and the resolution of BK disease (Prosser *et al*, 2008), respectively. In this study, we recorded CD4 and CD8 T-cell counts in peripheral blood mononuclear cells (PBMCs), in addition to CD4:CD8 ratios, as the effect of natalizumab on these parameters—albeit in CSF—has previously been documented (Stuve *et al*, 2006). Our PBMC data concur with those of Stuve and coworkers in that no significant effect was observed. However, in four of seven patients in whom BK virus reactivated, transient reductions in CD4 counts were observed at the onset of BK viruria. Suppression of virus replication in three of these patients corresponded with CD4 counts returning to baseline. The meaning of these transient fluctuations during natalizumab treatment is not known. However, it raises the possibility that even if natalizumab had an effect on CD4 counts of patients in our cohort, the immune system retained the capacity to respond appropriately to BKV

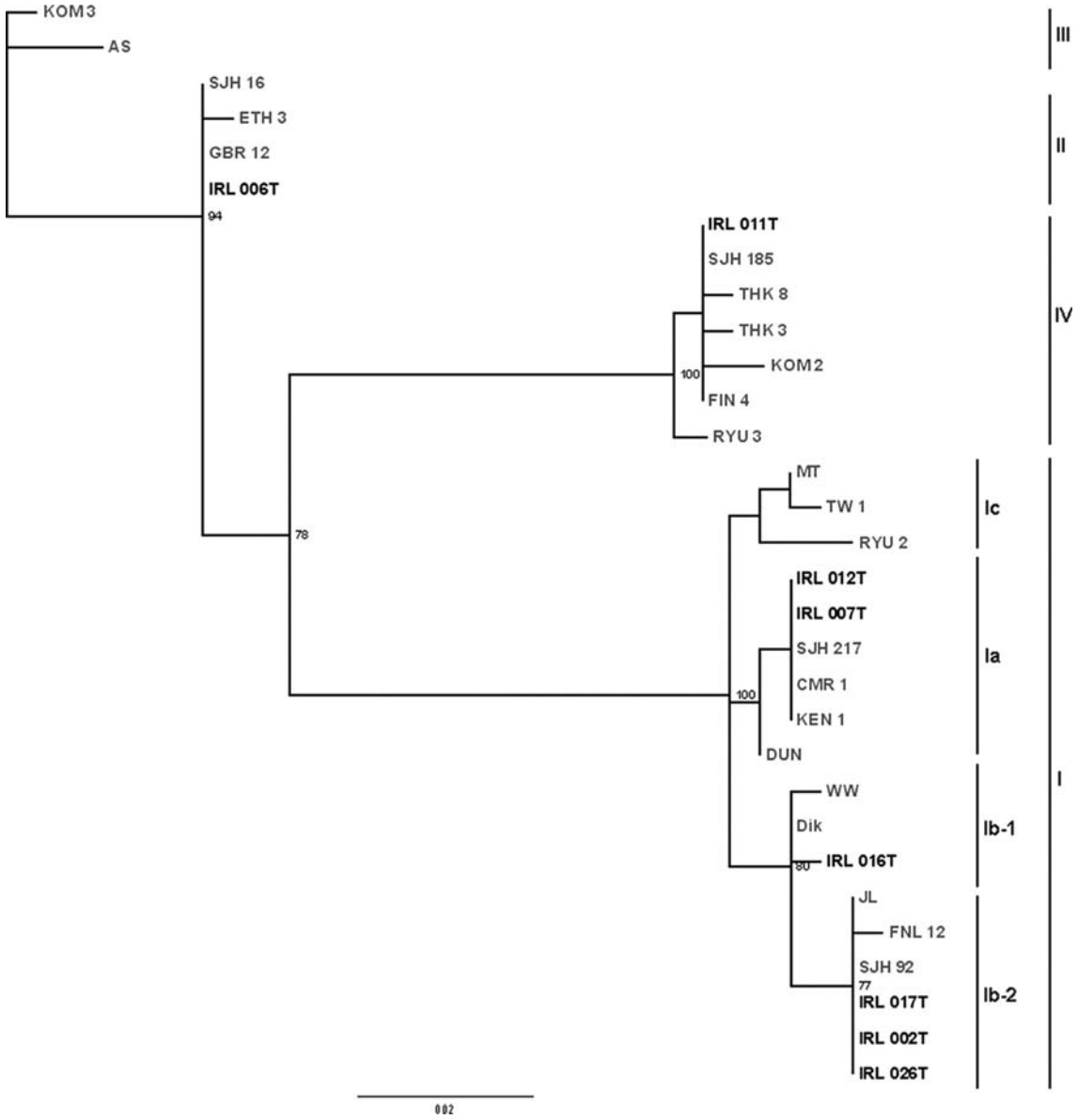

Figure 1 Maximum likelihood (ML) phylogenetic tree of BK virus reactivating in patients with MS on natalizumab therapy. The 287-bp BK virus typing sequences detected in eight patients with MS (corresponding to identifiers shown in bold) plus 23 reference sequences were used to reconstruct the ML tree. Subtypes and subtype I subgroups are indicated to the right of the tree. The numbers at nodes are bootstrap values (%) obtained for 1000 replicates. The geographic origins of the reference isolates were from Ireland (SJH-16, SJH-185, SJH-217, and SJH-92), the United States of America (DUN), Kenya (KEN-1), South Africa (WW), Cameroon (CMR-1), Ethiopia (ETH-3), The Netherlands (Dik and JL), Finland (FNL-12), the United Kingdom (AS and GBR-12), and Japan (KOM-2, KOM-3, MT, RYU-2, RYU-3, THK-8, and TW-1).

reactivation and suppress viral replication. Furthermore, as the CD4 levels may vary in an individual for various reasons, e.g., due to the time of day of sampling or delay in sample processing, these trends require further study and corroboration to determine clinical significance.

Studies of the immunomodulatory effect of natalizumab are ongoing. It has been shown to block α4β1/VCAM-1 (vascular cell adhesion molecule-1) interactions (Ransohoff, 2007). α4β1 is required to generate T and B cells from bone marrow progenitor cells in mice (Arroyo *et al*, 1996). This α4 integrin blockade may also mobilize JCV-infected B cells from bone marrow stores. However, due to insufficient contact with marrow stromal cells, these infected pre-B cells cannot effectively control viral replication (Ransohoff, 2005); and because natalizumab reduces CNS immune surveillance, reactivated JC virus may access the CNS via these B lymphocytes, or as cell-free virus (Sabath and Major, 2002), thereby leading to PML. This hypothesis is supported by the observation when PML occurs that JCV sequences in bone marrow and blood, rather than kidney or urine, most closely resemble those in CNS (Ransohoff, 2007). Furthermore, natalizumab has also been shown to mobilize CD34+

hematopoietic stem cells from bone marrow (Bonig *et al*, 2008; Zohren *et al*, 2008). Further study is required to ascertain whether the hypothesis advanced to explain the occurrence of PML in patients with MS receiving natalizumab may also explain the reactivation of BK virus demonstrated here. To date, studies of this nature (looking at BK reactivation) have been performed primarily in renal transplant recipients with polyomavirus nephropathy (PVN). Indeed, failure to mount a cellular immune response is linked to BKV replication and PVN development (Comoli *et al*, 2006). Furthermore, a strong cytotoxic T-lymphocyte (CTL) response appears to be associated with decreased BK viral load in blood and urine, and low anti-BKV antibody titers, playing an important role in the containment of BKV in renal transplant recipients with PVN (Chen *et al*, 2006). Anti-BKV serology and CTL responses were not measured in our study. Intriguingly, however, Chen *et al* (2006) also reported a high degree of epitope cross-recognition between BKV and corresponding JCV-specific CTLs, indicating the same population of cells is functionally effective against both viruses. Referring to reports of PML in patients treated with natalizumab, the authors suggested that—according to their data—if the CTLs were failing to prevent JCV reactivation, it was possible they would also fail to prevent BK reactivation. However, as BKV disease typically affects the kidney, a peripheral organ—in contrast to JC affecting the CNS, an immunologically privileged site in which viruses are relatively sheltered from the immune system—it may transpire that natalizumab's immunomodulatory effect will not be sufficient to lead to significant organ dysfunction even where BK reactivation occurs.

In the absence of clinically apparent renal impairment in the patients studied here, the implication of the BK reactivation observed remains to be established. These findings do, however, add to the increasing body of evidence demonstrating that natalizumab has a definite immunomodulatory effect. Whilst this effect may be pathogen group specific, i.e., increasing susceptibility to viruses—as opposed to bacteria or fungi—it does not appear to be polyomavirus specific, as HHV6 reactivation has been reported (Yao *et al*, 2008). That being said, no evidence for human herpes virus 5/cytomegalovirus (HHV5/CMV) reactivation was found in those patients reactivating BKV in our cohort.

BK virus has four known subtypes (I to IV) and VP1 typing studies and full length genome analysis have shown that subtype I predominates in all geographical regions; subtype IV occurs at lower rates and subtypes II and III occur rarely (Ikegaya *et al*, 2006; Zhong *et al*, 2009). Thus, the finding that two of our patients with MS reactivating BKV on natalizumab therapy were infected with subtypes II and IV is unexpected. In addition, the patient who was persistently viruric post treatment, and the only patient who was transiently viremic, reactivated the rare BK subtype II virus, suggesting further study is required to determine whether this genetic lineage is specifically associated with this patient group receiving natalizumab. We observed a significant genetic heterogeneity in the reactivated BKV subtypes in our MS patients receiving natalizumab. These did not correlate with the proposed geographic segregation of viral subtypes as all of our patients were Irish in origin. The genetic diversity in circulating BKV in Ireland substantiates a previous study on larger numbers of Irish samples showing that subtypes Ia, Ib1 and particularly subtype II (rarely observed elsewhere) are not infrequently detected in the population (Carr *et al*, 2006; Zhong *et al*, 2009). We saw no obvious correlation between gender, reactivating viral subtype, EDSS at baseline and the BK viral load.

As this was an observational study, limitations are inevitable and need to be addressed. Firstly, the study contains no control group. This issue was considered at the time of study planning and enrolment. It was concluded that as natalizumab had been established as the gold standard for care for those patients with MS eligible for the study, it would be unethical to withhold treatment from patients attending our service that satisfied the eligibility criteria. However, those patients in the present study that have discontinued natalizumab – for whatever reason – continue to be monitored for evidence of BK and JC reactivation as a *de facto* control group. Secondly, no samples of cerebrospinal fluid (CSF) were obtained from patients in this study, despite reports demonstrating reduced CD4:CD8 ratios in the CSF of patients with MS receiving natalizumab (Stuve *et al*, 2006). For the present study, the overriding aim was to identify a practical, inexpensive, non-invasive, and readily available means for the monitoring of patients for BK reactivation. Routine lumbar puncture at monthly intervals (as natalizumab is administered) is not in keeping with this goal. Thirdly, 21 patients in the present study had commenced natalizumab therapy (≤ 4 months) prior to enrolment. Although this is not ideal, we believe it would have been remiss to omit these patients, as – based on published reports of JC reactivation and PML in this patient cohort – the patients most likely to reactivate BK are those with the largest cumulative dose of natalizumab. Indeed, this decision has been vindicated as evidenced by the mean number of doses received by patients (11.2) prior to BK reactivation. However, some of this patient cohort could have reactivated BK earlier in their course of treatment and subsequently re-suppressed viral replication prior to enrolment in the study. Fourthly, one of the patients in whom BK viraemia was documented presented significant sampling problems. Briefly, this patient suffers with refractory epilepsy secondary to MS plaques, urinary incontinence, contractures, and requires full-time inpatient care. Despite consenting

to enrolment in the study, specimen collection was often distressing and so compliance with study protocol sub-optimal. The implications for the study are that despite confirmed viraemia on two successive samples in March and April 2007 a subsequent sample was not obtained until January 2008, at which BK was not detected in blood or urine. Whilst this does not impact significantly on the overall findings reported, as BK viraemia was uncommon, detailed study of the BK dynamics in this patient may have proven beneficial.

Conclusions

BKV reactivation occurs in patients with MS receiving natalizumab. The data presented here represent an important contribution to the novel and expanding field of knowledge surrounding the consequences of the (potential long-term) use of biological agents in the treatment of chronic immune-mediated illnesses and the possible infection-related adverse effects thereof. Furthermore, as the reported incidence of PML in this patient group is less than 1 in 1000, it is likely that if adverse events occur, they will be uncommon and remain uncovered during clinical trials. Therefore, a collaborative approach is vital, not only for research purposes, but primarily for the purpose of ensuring—insofar as is possible—the safety of patients being treated with this promising class of therapeutic agents. Similarly, it is important to establish that continued treatment with natalizumab in the presence of BK reactivation is safe and appropriate. Renal dysfunction has not occurred to date in these patients with BK virus reactivation. In addition, the absence of clinical manifestations of BK-related pathology in other organs may offer reassurance that, despite BK reactivation, continued therapy is not unsafe. This allows us to avoid unnecessary discontinuation of an effective therapy in patients with highly active MS. We therefore propose that patients with MS receiving natalizumab be regularly monitored, where possible, for evidence of BK virus reactivation, or at the very least for renal dysfunction.

Patients, methods, and materials

Since January 2007, all patients receiving natalizumab in a large university teaching hospital have been enrolled in an observational study to determine the presence of and predictors of polyomavirus reactivation. Fifty-seven patients have been enrolled to date. Twenty-one of these had commenced natalizumab therapy (for up to 4 months) prior to enrolment, and as such have no pretreatment blood and urine samples for analysis; however, complete data are available for the remaining 36 patients. Full informed consent was obtained for all participants, and the study received approval from the ethics committee of St. Vincent's University Hospital.

All eligible patients had active RRMS, and either rapidly evolving RRMS (defined by ≥ 2 disabling relapses in 1 year, with gadolinium enhancement on magnetic resonance imaging [MRI] or significant increase in T2 lesion load) or suboptimal response to standard immunomodulatory therapy (IMT) (with ≥ 1 relapse over 1 year). Of note, prior IMT had to be discontinued for at least 1 month prior to commencing natalizumab. At enrollment, each patient underwent the following: disability assessment using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983); MRI of the brain; baseline blood tests comprising complete blood count, coagulation screen, liver enzymes, and renal function; qualitative real-time polymerase chain reaction (PCR) to monitor for JC and BK DNA in serum and urine (and subsequent quantitative real-time PCR for each polyomavirus in positive specimens); measurement of absolute CD4 and CD8 cell counts, along with calculation of CD4:CD8 ratios in peripheral blood mononuclear cells (PBMCs). All blood and urine tests were performed monthly for the first 3 months after enrolment and 3-monthly thereafter and CD4 and CD8 ratios were determined by flow cytometry by standard techniques.

Total nucleic acids were extracted from 140 µl of urine and serum specimens using the QIAmp viral RNA minikit (Qiagen), exactly as per manufacturer's instructions, and eluted in a final volume of 60 µl AVE buffer. Qualitative real-time PCR for JCV/BKV DNA was carried out on the Lightcycler 1.0 instrument (Roche Molecular Diagnostics) with the FastStart DNA master hybridization probes kit (Roche Molecular Biochemicals) as described (Whiley *et al*, 2001). Negative and positive controls were included in each assay run. The Lightcycler PCR cycling parameters were as follows: 10 min incubation at 95°C for FastStart *Taq* DNA polymerase activation followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 20 s with data acquisition in the annealing phase. Following amplification, capillaries were heated from 55°C to 95°C at a rate of 0.1°C/s and nucleotide sequence polymorphisms between the JCV and BKV VP2 genes allow discrimination of the polyomaviruses as amplification of the BKV PCR amplicons resulted in a shift in melting temperature (~5°C) compared to the results for the JCV amplicons. To generate a standard curve for real-time quantitation of BKV from positive specimens identified by the qualitative PCR for JCV and BKV described above, primers targeting the conserved large tumor (T) antigen gene (LTAg) primers: BKV-TAg.for 5′-AGCAGGCAAGGGTTCTATTAC-TAAAT-3′; BKV-TAg.rev 5′-GAAGCAACA GCA-GATTCTCAACA-3′ were used to amplify a 131-bp fragment of the BK virus (Dun strain) genome using a

HotStar HiFidelity DNA polymerase (Qiagen) with the following reaction conditions: 95°C for 5 min, 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. These amplicons were purified using a PCR purification kit (Qiagen) and quantified on a Nanodrop ND-100 spectrophotometer from which the target concentration in copies per microliter was determined. The quantitative PCR assay is a modification of a previously published assay (Hirsch *et al*, 2001), employing the modified minor groove-binding (MGB) probe labeled at the 5′ end with 6-carboxyfluorescin (FAM): FAM-AGACCCTAAAGACTTTCCCT-MGB. The assay was run on the ABI7300 SDS (Applied Biosystems). Each 25-μl reaction contained 1× Universal master mix (Applied Biosystems), 300 nM forward and reverse primers, and 200 nM of the MGB probe, respectively. Five microliters of nucleic acid eluate were added to each reaction and the cycling parameters were as follows: 50°C×2 min (1 cycle); 95°C×10 min (1 cycle); 95°C×15 s, 60°C×1 min (40 cycles) with fluorescence detection in the annealing/extension phase. The linear dynamic range of the BKV quantitative PCR was 10^{10} to 10^{2} copies of the BKV LTAg amplicon and the limit of detection is between 10 and 100 copies of target. BKV VP1 subtyping/noncoding control region (NCCR) characterization were performed exactly as described previously (Carr *et al*, 2006). Quantitative cytomegalovirus (CMV) DNA testing was carried out using the CMV TM PCR Kit (Artus) as per manufacturer's instructions on plasma samples from patients with BK reactivation.

GenBank accession numbers

The following nucleotide sequence accession numbers from unique BK virus VP1 typing regions have been deposited in GenBank: 006T (FJ639173), 011T (FJ639174), 012T (FJ639175), 016T (FJ639176), and 017T (FJ639177).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Arroyo AG, Yang JT, Rayburn H, Hynes RO (1996). Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. *Cell* **85:** 997–1008.

Arthur RR, Shah KV, Baust SJ, Santos GW, Saral R (1986). Association of BK viruria with hemorrhagic cystitis in recipients of bone marrow transplants. *N Engl J Med* **315:** 230–234.

Behzad-Behbahani A, Klapper PE, Vallely PJ, Cleator GM, Khoo SH (2004). Detection of BK virus and JC virus DNA in urine samples from immunocompromised (HIV-infected) and immunocompetent (HIV-non-infected) patients using polymerase chain reaction and microplate hybridisation. *J Clin Virol* **29:** 224–229.

Bonig H, Wundes A, Chang KH, Lucas S, Papayannopoulou T (2008). Increased numbers of circulating hematopoietic stem/progenitor cells are chronically maintained in patients treated with the CD49d blocking antibody natalizumab. *Blood* **111:** 3439–3441.

Carr MJ, McCormack GP, Mutton KJ, Crowley B (2006). Unique BK virus non-coding control region (NCCR) variants in hematopoietic stem cell transplant recipients with and without hemorrhagic cystitis. *J Med Virol* **78:** 485–493.

Chen Y, Trofe J, Gordon J, Du Pasquier RA, Roy-Chaudhury P, Kuroda MJ, Woodle ES, Khalili K, Koralnik IJ. (2006). Interplay of cellular and humoral immune responses against BK virus in kidney transplant recipients with polyomavirus nephropathy. *J Virol* **80:** 3495–505.

Comoli P, Binggeli S, Ginevri F, Hirsch HH. (2006). Polyomavirus-associated nephropathy: update on BK virus-specific immunity. *Transpl Infect Dis* **8:** 86–94.

Egli A, Infanti L, Dumoulin A, Buser A, Samaridis J, Stebler C, Gosert R, Hirsch HH (2009). Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis* **199:** 837–846.

FDA (2008). Natalizumab (marketed as Tysabri) Information.

Gardner SD, Field AM, Coleman DV, Hulme B (1971). New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet* **1:** 1253–1257.

Hirsch HH, Mohaupt M, Klimkait T (2001). Prospective monitoring of BK virus load after discontinuing sirolimus treatment in a renal transplant patient with BK virus nephropathy. *J Infect Dis* **184:** 1494–1495.

Ikegaya H, Saukko PJ, Tertti R, Metsarinne KP, Carr MJ, Crowley B, Sakurada K, Zheng HY, Kitamura T, Yogo Y (2006). Identification of a genomic subgroup of BK polyomavirus spread in European populations. *J Gen Virol* **87:** 3201–3208.

Kalvatchev Z, Slavov S, Shtereva M, Savova S (2008). Reactivation of Polyomavirus hominis 1 (BKV) during pregnancy and the risk of mother-to-child transmission. *J Clin Virol* **43:** 328–329.

Kappos L, Bates D, Hartung HP, Havrdova E, Miller D, Polman CH, Ravnborg M, Hauser SL, Rudick RA, Weiner HL, O'Connor PW, King J, Radue EW, Yousry T, Major EO, Clifford DB (2007). Natalizumab treatment for multiple sclerosis: recommendations for patient selection and monitoring. *Lancet Neurol* **6:** 431–441.

Khalili K, White MK, Lublin F, Ferrante P, Berger JR (2007). Reactivation of JC virus and development of PML in patients with multiple sclerosis. *Neurology* **68:** 985–990.

Knowles WA (2002). The epidemiology of BK virus and the occurrence of antigenic and genomic subtypes. In: *Human polyomaviruses: molecular and clinical perspectives*. Khalili K, Stoner GL (eds). New York: Wiley-Liss, pp 527–558.

Kurtzke JF (1983). Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* **33:** 1444–1452.

Markowitz RB, Thompson HC, Mueller JF, Cohen JA, Dynan WS (1993). Incidence of BK virus and JC virus viruria in human immunodeficiency virus-infected and -uninfected subjects. *J Infect Dis* **167:** 13–20.

Nishimoto Y, Zheng HY, Zhong S, Ikegaya H, Chen Q, Sugimoto C, Kitamura T, Yogo Y (2007). An Asian origin for subtype IV BK virus based on phylogenetic analysis. *J Mol Evol* **65:** 103–111.

Polo C, Perez JL, Mielnichuck A, Fedele CG, Niubo J, Tenorio A (2004). Prevalence and patterns of polyomavirus urinary excretion in immunocompetent adults and children. *Clin Microbiol Infect* **10:** 640–644.

Prosser SE, Orentas RJ, Jurgens L, Cohen EP, Hariharan S (2008). Recovery of BK virus large T-antigen-specific cellular immune response correlates with resolution of bk virus nephritis. *Transplantation* **85:** 185–192.

Ransohoff RM (2005). Natalizumab and PML. *Nat Neurosci* **8:** 1275.

Ransohoff RM (2007). "Thinking without thinking" about natalizumab and PML. *J Neurol Sci* **259:** 50–52.

Reploeg MD, Storch GA, Clifford DB (2001). Bk virus: a clinical review. *Clin Infect Dis* **33:** 191–202.

Rodrigues C, Pinto D, Medeiros R (2007). Molecular epidemiology characterization of the urinary excretion of polyomavirus in healthy individuals from Portugal—a Southern European population. *J Med Virol* **79:** 1194–1198.

Rosen S, Harmon W, Krensky AM, Edelson PJ, Padgett BL, Grinnell BW, Rubino MJ, Walker DL (1983). Tubulointerstitial nephritis associated with polyomavirus (BK type) infection. *N Engl J Med* **308:** 1192–1196.

Sabath BF, Major EO (2002). Traffic of JC virus from sites of initial infection to the brain: the path to progressive multifocal leukoencephalopathy. *J Infect Dis* **186 (Suppl 2):** S180–S186.

Schaffer K, Sheehy N, Coughlan S, Bergin C, Hall WW (2006). JC virus in the Irish population: significant increase of genotype 2 in immunocompromised individuals. *J NeuroVirol* **12:** 39–46.

Stuve O, Marra CM, Bar-Or A, Niino M, Cravens PD, Cepok S, Frohman EM, Phillips JT, Arendt G, Jerome KR, Cook L, Grand'Maison F, Hemmer B, Monson NL, Racke MK (2006). Altered CD4 + /CD8+ T-cell ratios in cerebrospinal fluid of natalizumab-treated patients with multiple sclerosis. *Arch Neurol* **63:** 1383–1387.

Sundsfjord A, Flaegstad T, Flo R, Spein AR, Pedersen M, Permin H, Julsrud J, Traavik T (1994). BK and JC viruses in human immunodeficiency virus type 1-infected persons: prevalence, excretion, viremia, and viral regulatory regions. *J Infect Dis* **169:** 485–490.

Whiley DM, Mackay IM, Sloots TP (2001). Detection and differentiation of human polyomaviruses JC and BK by LightCycler PCR. *J Clin Microbiol* **39:** 4357–4361.

Wong AS, Chan KH, Cheng VC, Yuen KY, Kwong YL, Leung AY (2007). Relationship of pretransplantation polyoma BK virus serologic findings and BK viral reactivation after hematopoietic stem cell transplantation. *Clin Infect Dis* **44:** 830–837.

Yao K, Gagnon S, Akhyani N, Williams E, Fotheringham J, Frohman E, Stuve O, Monson N, Racke MK, Jacobson S (2008). Reactivation of human herpesvirus-6 in natalizumab treated multiple sclerosis patients. *PLoS ONE* **3:** e2028.

Yousry TA, Major EO, Ryschkewitsch C, Fahle G, Fischer S, Hou J, Curfman B, Miszkiel K, Mueller-Lenke N, Sanchez E, Barkhof F, Radue EW, Jager HR, Clifford DB (2006). Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. *N Engl J Med* **354:** 924–933.

Zhong S, Randhawa PS, Ikegaya H, Chen Q, Zheng HY, Suzuki M, Takeuchi T, Shibuya A, Kitamura T, Yogo Y (2009). Distribution patterns of BK polyomavirus (BKV) subtypes and subgroups in American, European and Asian populations suggest co-migration of BKV and the human race. *J Gen Virol* **90:** 144–152.

Zhong S, Zheng HY, Suzuki M, Chen Q, Ikegaya H, Aoki N, Usuku S, Kobayashi N, Nukuzuma S, Yasuda Y, Kuniyoshi N, Yogo Y, Kitamura T (2007). Age-related urinary excretion of BK polyomavirus by nonimmunocompromised individuals. *J Clin Microbiol* **45:** 193–198.

Zohren F, Toutzaris D, Klärner V, Hartung HP, Kieseier B, Haas R (2008). The monoclonal anti-VLA-4 antibody natalizumab mobilizes CD34+ hematopoietic progenitor cells in humans. *Blood* **111:** 3893–3895.