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Influence of JC virus coding region genotype on risk of multiple sclerosis and progressive multifocal leukoencephalopathy

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Two features of the biology of JC virus make it a particularly suitable candidate for an agent in MS-like disease: its neurotropic capability targeting glial cells as evidenced in progressive multifocal leukoencephalopathy lesions, and its capacity for latency and persistence as illustrated by its behaviour in the kidney. JC virus is chronically or intermittently excreted in the urine by some 40% of the population. The existence of JC virus in multiple coding-region genotypes provides a unique approach to the study of JC virus-induced neurological disease. We have previously shown that a genotype originating in Asia but also present in Europe and the US, called Type 2B, is more frequently found in PML brain than expected based on its prevalence in urine samples from a control population. In contrast, we find that the excretion of JCV in MS patients is similar in both genotype and frequency to that of control individuals, and appears to be regulated by factors unrelated to those that control CNS disease activity. *Journal of NeuroVirology* (2000) **6,** S101–108.

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Introduction

In 1958, Åström *et al* described a CNS demyelinating disease of unknown aetiology associated with chronic lymphocytic leukaemia and lymphoma which showed characteristic oligodendrocytopathy and giant astrocytes (Åström *et al*, 1958). They named this new pathological entity progressive multifocal leukoencephalopathy (PML). The association of the human polyomavirus called JC virus (JCV) with this demyelinating disease dates from 1971 when Padgett, Walker and colleagues (Padgett *et al*, 1971) cultured the agent which had first been observed in PML lesions with the electron microscope in 1965 (ZuRhein and Chou, 1965). The virus was named JC and this strain (Mad1) was completely sequenced in 1984 (Frisque *et al*, 1984). The circular double-stranded DNA virus has a 5.1-kb genome that is bidirectionally transcribed. In the PML brain oligodendrocytes are lytically infected causing the pathognomonic foci of demyelination. Astrocytes are abortively infected (Åström and Stoner, 1994).

PML is the best example of a human viral primary demyelinating disease. The similarity of PML lesions to those of MS was first noted by Åström *et al* in their original description. They observed that 'the disease closely resembles multiple sclerosis' in its basic aspects, but also showed that the unique features of PML make the two diseases readily distinguishable (Åström *et al*, 1958).

It is clear that MS lesions do not have easily detectable levels of JCV (Buckle *et al*, 1992; Stoner *et al*, 1986), and the viral mechanisms involved, if any, must be quite different from those seen in PML. However, reports of direct studies of MS brain and CSF for evidence of JCV have been relatively few. One report showed evidence for viral DNA in 9% of 121 samples of MS CSF samples using a nested PCR, while 30 controls with other neurological diseases and 24 non-neurological controls were all negative (Ferrante *et al*, 1998).

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The molecular era of neurovirology has stimulated new thinking about other less direct mechanisms of cell damage in which non-structural early viral proteins might be implicated, without the productive cytolytic viral infection that occurs in PML. In this way a similar disease could be caused by the same virus but employing a very different mechanism (Stoner, 1991, 1993; Stoner *et al*, 1988). The mechanisms that can be envisioned involving nonstructural early proteins of JCV include metabolic and developmental effects of these DNA-binding phosphoproteins (Tretiakova *et al*, 1999; Haas *et al*, 1994; Trapp *et al*, 1988), or the possible targeting by the immune system of viral T antigens (Tevethia *et al*, 1998; Lill *et al*, 1992), a fraction of which appears on the cell surface. Virus present at extremely low levels, e.g., one copy per glial cell, would suffice for these mechanisms. Further, the death of infected cells might lead to eventual loss of virus from lesions with other mechanisms, presumably immunological in nature, taking over. For these reasons such an occult agent might be difficult to detect.

Infection with the archetypal JCV is thought to take place early in childhood. Members of the immediate family or individuals in the close social environment are likely the major source of infecting virus. Why seroconversion does not usually take place until adolescence remains unclear (Walker and Padgett, 1983). The upper respiratory tract is a likely site of primary infection (Monaco *et al*, 1998a). JCV DNA can sometimes be detected in peripheral blood lymphocytes that may serve as a carrier to different tissues (Jensen and Major, 1999; Monaco *et al*, 1998b). In the kidney JCV can establish a persistent infection from which the virus may be continuously shed in the urine (Agostini *et al*, 1996b; Kitamura *et al*, 1990). Rates of excretion of viral DNA in the urine of adults vary with different population groups from as low as 20% in Africans to as high as 80% in Asian and related populations (Agostini *et al*, 1999). The frequency of JCV excretion seems not to be linked to immunological parameters in HIV-1-infected individuals (Sundsfjord *et al*, 1994; Markowitz *et al*, 1993). This is in contrast to the urinary excretion of BK virus (BKV), the other human polyomavirus, for which the frequency and rate of urinary excretion do depend on the immune status of the individual (Sundsfjord *et al*, 1994; Markowitz *et al*, 1993).

The ready availability of JCV for molecular epidemiological study has revealed distinct coding-region genotypes associated with different geographical regions of the world. In general, coding region variation is probably a result of coevolution of the virus with different human populations. A total of seven geographically based genotypes and additional subtypes have been identified to date (Agostini *et al*, 1999). The major genotypes of the world are present in the US with its diverse population background (Jobes *et al*, 1998; Agostini *et al*, 1996b). These genotypes provide a novel approach to the analysis of neurological diseases caused (or potentially caused) by JCV.

In previous studies we have shown that JCV coding-region genotypes are distributed in PML brain differently from their distribution in the urines of an ethnically comparable control population (Agostini *et al*, 1997a, 1998a). The JCV genotype known as Type 2B was significantly elevated in PML brain compared to the numbers in control urines. At the same time, Type 4 (Western European) was under-represented in PML brains.

Here we focus on an indirect approach to assessing a possible role for JCV in MS by asking whether distribution of JCV genotypes in the urine of MS patients in the US differs from that in a matched control group. This approach is based on the knowledge that JCV exists as geographically based genotypes including Types 1 and 4 in the US and Europe, Types 2 and 7 in Asia, and Types 3 and 6 in Africa (Jobes *et al*, 1998; Sugimoto *et al*, 1997; Ou *et al*, 1997; Agostini *et al*, 1996b; Guo *et al*, 1996). The non-European genotypes are also found in the US, but their representation varies with ethnicity. We have examined urines of 95 MS patients, some collected serially, for JCV DNA, and have found that patients may be either excreters, non-excreters or intermittent excreters, and we have sought a relationship of this excretion status to MS disease activity.

In addition to the evolutionary coding region genotypes of JCV, the viral genome can vary in a fundamentally different way. The non-coding regulatory region of the circulating virus surrounds the origin of replication (*ori*) and exists mainly in an archetypal configuration that can be rearranged in the transcriptional control portion by deletions and duplications likely occurring within the lifetime of the infected individual (Agostini *et al*, 1997b, 1998b; Ault and Stoner, 1993). These varied forms, which follow no simple pattern, are found mainly in PML brain tissue. Whether these rearranged PML strains, e.g., the prototype Mad1 strain, can be transmitted from one individual to another is still unclear. Here we have also looked at JCV variation in the archetypal regulatory region. We have studied MS patients from both the US and Hungary. These results have been reported in part previously (Agostini *et al*, 1998b).

In a direct test of the hypothesis that JCV plays a role in MS, we have also examined CSF samples from 42 MS patients and 27 controls for JCV DNA.

Results

JCV excretion in urine

A total of 94 patients with multiple sclerosis were tested for JC viruria by PCR. The clinical course of the disease in these patients was relapsing-remit-

ting in 14%, relapsing-progressive in 36%, and chronic-progressive in 50%. Of the first urine samples obtained from this group, 38 (40%) tested positive for viral DNA by PCR using the JLP-15 and 16 fragment within the VP1 gene (Table 1). To determine whether disease activity or different treatment schemes were associated with JC viruria, urinary samples of 54 of the 94 MS patients were tested two or more times (Table 2). From these 54 patients a total of 206 samples were collected over a period of 2–138 months with an average collection period of 23 months and a median of 15 months. Patients were tested from two to nine times for urinary excretion of JCV with a median of four tests per patient.

Three types of JCV excretion patterns could be distinguished: chronic excreters (positive in all samples tested), intermittent excreters (positive in one or more but not all of the samples tested), and non-excreters (negative in all samples tested). Of the 54 serially tested patients, 24 (44%) were chronic excreters, 11 (20%) were intermittent excreters, and 19 (35%) were non-excreters. The association of excretion pattern and course of MS disease is shown in Table 2. One relapsing-progressive patient excreted JCV each of nine times tested over 18 months. Each of the JCV genotypes identified was Type 1B. Another relapsing-progressive patient excreted Type 1B each of six times tested over 22 months. A third relapsing-progressive patient, a Mexican-American, excreted Type 2A each of six times tested over 18 months.

Some intermittent excreters were predominantly positive; for example, a relapsing-remitting patient who excreted Type 2B in six of eight samples tested over 16 months. Others were positive for JCV only once, as in a relapsing-remitting patient tested eight times over 18 months.

The disease activity as determined by clinical assessment charts (EDSS and FSS) in the 11 patients with intermittent excretion of JCV were not associated. There was no relationship between disease activity or progression and excretion status. Chronically excreting patients remained excreters through periods of inactivity as well as progression or clinical relapse. Similarly, non-excreters remained negative even during fluctuations in clinical parameters. In a few cases intermittent excreters appeared to show episodes of excretion in temporal relation to clinical exacerbation, but more frequent testing for urinary JCV would be required to establish whether any consistent association exists.

JCV genotype distribution in urine from MS patients

The frequency of different JCV genotypes in urine in relation to MS clinical course is shown in Table 1. There was no statistically significant preference of any genotype for a particular MS clinical course. However, some ethnic associations were evident. The large majority of European Americans excreted Type 1. The Type 3 strains (African) appeared in the urine of two African-Americans and a Hispanic MS patient. Several of the nine Type 2A strains, thought

Table 1 Type distribution of JCV-positive MS patients

Clinical course	*No. patients*	*JCV-positive (first test)*	*JCV-positive (overall)*	*JCV genotypes*[a]						
				Type 1	*Type 2 A/C*	*Type 2B*	*Type 3*	*Type 4*	*Type 5*	*ND*[b]
Relapsing-remitting	13	6 (46%)	7 (54%)	2	2	1			1	1
Relapsing-progressive[c]	34	19 (56%)	23 (68%)	11	6	1	1	4		1
Chronic-progressive[c]	47	13 (28%)	16 (34%)	10	3	1	2	1		
Total	94	38 (40%)	46 (49%)	23	11	3	3	5	1	2

[a]See Agostini *et al* (1999) for definitions of JCV genotypes. [b]Not determined. [c]Two patients, one relapsing-progressive and one chronic-progressive, were co-infected with two different JCV genotypes. Thus, one extra JCV strain was identified in each group.

Table 2 JCV excretion in MS patients tested more than once

Clinical course	*No. of patients (total)*	*No. tested multiple times*	*No. chronic excreters*[a]	*No. intermittent excreters*	*No. non-excreters*
Relapsing-remitting	13	12[b]	4 (33%)	2 (17%)	6 (50%)
Relapsing-progressive	34	29[c]	17 (59%)	5 (17%)	7 (24%)
Chronic-progressive	47	13[d]	3 (23%)	4 (31%)	6 (46%)
Total	94	54	24 (44%)	11 (20%)	19 (35%)

[a]No. patients excreting virus every time tested. [b]Relapsing-remitting patients tested two to eight times (mean 4.4). [c]Relapsing-progressive patients tested two to nine times (mean 4.1). [d]Chronic-progressive patients tested two to six times (mean 3.0).

Table 3 Distribution of JCV genotypes between MS cohort and controls

Cohort	Total no.	JCV genotypes						
		Type 1	Type 2 A/C	Type 2B	Type 3	Type 4	Type 5	Type 6
All MS	46	23	11	3	3	5	1	0
Controls								
PML	53	25	6	19[a]	0	2	0	1
Clin[b]	45	29	6	2	1	7	0	0
HIV	32	13	9	3	1	6	0	0
Total	176	90	32	27	5	20	1	1

[a]$P<0.001$. [b]General medical clinic patients or healthy volunteers.

to be northeast Asian in origin, were found in patients with Hispanic surnames. Further study will be required to determine whether Type 2A is elevated in the Hispanic group.

The genotype distribution in MS patient urines and those of HIV-1-infected and uninfected control urines, as well as the genotype profile in PML brains is shown in Table 3. There was no significant difference between genotypes excreted by MS patients and the non-neurological control groups either separately or combined. However, as reported previously, the frequency of JCV Type 2B is significantly elevated in PML brain compared to either the MS cohort or the other control groups (Agostini *et al*, 1998a).

Regulatory region alterations in JCV from urine of MS patients

From 13 JCV-positive Hungarian MS patients and 12 JCV-positive paired controls we could amplify the regulatory region of a total of 13 JCV samples. One MS patient showed a mutation at position 256 (archetypal numbering) of C to T. The others were polymorphic at position 108 (three controls and one MS patient had 'A', while five controls and four MS patients had 'G'). The four probands with 108-A were genotyped as Type 1A, whereas the nine with 108-G were divided. Three were Type 1A, and 5 were Type 1B. All regulatory regions had 'A' at position 217.

As previously described (Agostini *et al*, 1998b), several MS patients in the US showed changes in the regulatory region sequence flanking the third NF-1 site (Figure 1). One of these involved a deletion of seven base pairs with a duplication of six base pairs from the first half of the site (#224). In another (#230) nucleotides 221 and 222 were deleted, with nucleotide 219 mutated from G to A. Both of these strains were genotyped as Type 2.

Drug treatment and JCV excretion in urine of MS patients

Of the 54 serially tested patients 11 were treated with interferon beta-1b during the testing period from 3 months up to 23 months. Other treatment regimens in individual cases included azathioprine and methotrexate. In the interferon beta-1b group four were chronic excreters, two were intermittent excreters, and five were non-excreters of JCV DNA. Patients who were intermittent excreters did not change their excretion pattern when started on interferon, nor did it induce or stop viral excretion in the other nine patients. This finding is consistent with the evidence that urinary excretion of BKV, but not JCV, depends on the immune status of the host (Sundsfjord *et al*, 1994; Markowitz *et al*, 1993).

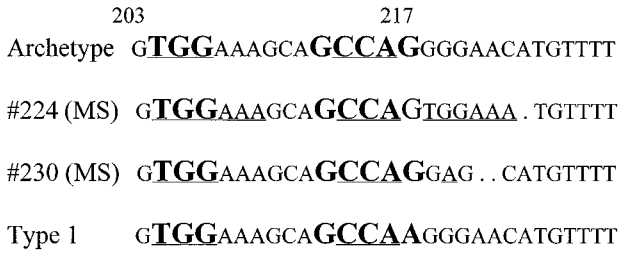

Figure 1 Changes in the regulatory region sequence flanking the third NF-1 site of two MS patients. The palindromic portion of the consensus sequence $(TGGA/C(N)_5GCCAA)$ (Blomquist *et al*, 1999) is underlined in bold. Strain #224 was from an Hispanic patient (male/age 72). Strain #230 was from an African-American patient (male/age 44).

Failure to detect JCV DNA in CSF of MS patients

Eighty-four CSF samples were tested for the presence of JCV DNA by PCR with primers JLP-15 and 16. The series included 57 samples from 42 MS patients and 27 samples from 27 patients with other neurological diseases (19 with Alzheimer's disease or dementia, three with Parkinson's disease, one with Pick's disease and four with acute neurological symptoms). Initially, all samples were coded and tested blindly. The code was broken after all the CSF samples had been analyzed by PCR for JCV DNA in the VP1 fragment. None of the CSF samples tested positive for JCV DNA (JLP-15 and 16 fragment). In our hands these primers provide the most sensitive detection of JCV DNA in clinical samples.

Discussion

Viruses could act in several different ways to promote altered glial cell function and loss of myelin. In PML brain JCV lytically infects oligoden-

drocytes, destroying their ability to maintain myelin. In abortive JCV infections, such as occur in astrocytes, protein expression is largely limited to the early, non-structural proteins of JCV, i.e., large and small T-antigens. These DNA-binding proteins expressed in the nucleus, are also transported at low levels to the cell surface, potentially inviting immunologically mediated attack (Stoner *et al*, 1988). Finally, viral regulatory proteins would only need to perturb glial cell metabolism to interfere with expression of their highly specialized myelin forming function (Tretiakova *et al*, 1999).

Underlying the present studies is the hypothesis that JCV latently infects the brain in some individuals (Stoner, 1993), and that this latent virus with the potential for abortive reactivation, is a candidate for a role in demyelinating diseases of unknown aetiology such as multiple sclerosis. Furthermore, it is hypothesised that different genotypes of JCV would behave differently in the brain of MS patients, as has been shown for PML (Agostini *et al*, 1997a, 1998a). JCV Type 2B is associated with a higher risk of PML, whereas other genotypes such as Type 2A and Type 4 have a lower prevalence in PML patients. It is proposed that if JCV plays a key role in MS as it does in PML, there may be a similar discernible skewing of the genotype distribution, this time among the excreted genotypes. As a direct test of this hypothesis, we have also examined CSF of both MS patients and those with other neurological diseases for evidence of JCV DNA by PCR.

Our hypothesis was testable in these patients because 44% of MS patients were chronic excreters of JCV and 20% excreted the virus intermittently. One patient excreted JCV at each of the nine times he was tested over the course of 18 months. Non-excreters made up 35% of the MS patients. The immunological or other basis for these distinctions is unclear. We found no indication of an association between MS disease activity (stationary, progression, or clinical relapse) and virus excretion, although more detailed study will be required to rule out any association between intermittent excretion and clinical relapse. This suggests that the immunological response and inflammatory mediators thought to underlie disease activity in the CNS are unrelated to the factors controlling virus excretion from the kidney. The urinary excretion may merely reflect the extent of kidney infection, in particular that of the tubular epithelial cells, with heavily infected kidneys excreting continuously, and uninfected kidneys never excreting virus. Intermittent excreters would represent mild renal tubular epithelial cell infection with minimal release of JCV DNA that is sometimes below the limits of detection. In most patients the genotype of the virus excreted was constant, but in one African-American MS patient with apparent double infection the genotype switched within 1 week from Type 3 to a mixed Type 2A/Type 3 infection (Agostini *et al*, 1996a). Since BKV excretion is more closely tied to the immunological status of the host (Sundsfjord *et al*, 1994; Markowitz *et al*, 1993), BKV excretion may prove to be a more sensitive viral indicator of changing systemic immunological status in MS patients.

The present results failed to support the hypothesis that JCV genotypes are represented differently in MS patients than in an ethnically comparable control group. This contrasts with the findings in PML tissue in which the Eurasian genotype known as Type 2B is significantly over-represented (Agostini *et al*, 1997a, 1998a).

A direct search by PCR for JCV DNA in CSF of MS patients failed to provide evidence of viral involvement in the MS brain. While JCV DNA was below the limits of detection of our assay, we do not rule out the possibility that a more sensitive assay or a larger series might reveal the presence of viral DNA. It is possible that JCV DNA in the MS CSF might be limited to particular stages of disease not well represented in our sample. Finally, as noted above, virus-infected cells might be eliminated by the disease process itself.

An intriguing observation in these studies was the finding that the regulatory region of JCV in some MS patients has minor alterations, some of which are associated with the third NF-1 binding site. The binding of the transcriptional factor NF-1 site is thought to be important for the glial-specific expression of JCV (Amemiya *et al*, 1992). The possibility exists that changes in DNA sequence flanking sites such as these can affect their availability to transcriptional activators, possibly by influencing the local histone–DNA contacts and thus the accessibility of the NF-1 site in a nucleosome context (Blomquist *et al*, 1999).

While the role of JCV, if any, in the aetiology of MS remains elusive, the aetiologic agent of PML illustrates much about the potential non-lytic mechanisms by which a virus might be involved in primary CNS demyelination.

Materials and methods

Urine samples

A total of 256 urinary samples collected from 94 MS patients were obtained from the National Neurological Research Specimen Bank. Of these a total of 206 were serial samples from 54 patients. The testing frequency ranged from two to nine times with a median of four tests per individual. Most patients were observed at intervals of 6 months over a period of 18 months. For some patients a sample taken 10 years earlier was available, extending the period of observation to approximately 11.5 years. Methods and results on those initial studies were published previously (Stoner *et al*, 1996).

Urine samples from 30 MS patients and 30 controls were obtained from Budapest. Their genotypes (Type 1A and Type 1B) were reported previously (Stoner *et al*, 1998). Here we report the analysis of changes in the archetypal JCV regulatory region.

Clinical assessments in patients at VA Medical Center-West Los Angeles utilised the Kurtzke Expanded Disability Status Scale (EDSS), and the Functional System Scale (FSS) as described previously (Syndulko *et al*, 1996).

Control individuals
As controls, 77 individuals without MS or PML were studied. Of these, 32 were HIV-1-positive. These controls were ethnically comparable to the patients in the MS cohort. The ethnic distribution of both MS and control cohorts included a majority of Europeans, with appropriate representation by African-Americans and Hispanics. The PML patients included in this study and the methods by which they were genotyped have been described (Agostini *et al*, 1997a, 1998a).

DNA extraction of urine for PCR
Urine (10–15 ml) stored at 4°C was centrifuged at low speed and the cell pellet washed once with sterile phosphate buffered saline without Mg^{2+}. The cells were recentrifuged and the pellet resuspended in 200 μl lysis buffer containing 50 mM Tris, 1 mM EDTA, 0.45% NP40, and 0.45% Tween20, adjusted to pH 8.0, and digested at 55°C with proteinase K at a final concentration of 0.2 mg/ml for a minimum of 3 h. The reaction was stopped by boiling for 10 min.

PCR on urinary DNA
Primers JLP-15 and 16 were utilised as described (Agostini *et al*, 1997c). The positive samples were then subjected to amplification with primers JRR-25 and 28 for analysis of changes in the archetypal regulatory region sequence (Agostini *et al*, 1997b).

PCR on CSF
CSF (400 μl) was extracted using the Qiagen Tissue Kit (Kit no. 29304) according to the manufacturer's protocol. Primers JLP-15 and 16 were used to amplify a 215-bp fragment in the 5′-end of the VP1 gene for genotyping (Agostini *et al*, 1997c). PCR utilised Turbo Pfu DNA polymerase (Stratagene) with the specificity of the reaction enhanced with Perfect Match (Stratagene). Initial denaturation at 95°C for 5 min was followed by 50 cycles of 95°C for 1 min, 62°C for 1.5 min, 72°C for 1 min, followed by a 10 min extension at 72°C and then 4°C for termination.

Direct cycle sequencing
Direct cycle sequencing was performed as previously described (Agostini *et al*, 1997c).

Statistical analysis
The methods for statistical analysis have been described (Agostini *et al*, 1997a).

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