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JC virus in the Irish population: Significant increase of genotype 2 in immunocompromised individuals

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The human polyomavirus JC virus (JCV) is ubiquitous and can be shed in the urine of more than 40% of the healthy population. Amplification and sequencing of JCV from urine has allowed a distinctive map of the distribution of JCV genotypes worldwide. To define the frequency of JCV urinary excretion and genotype distribution in Ireland, urines from 121 healthy individuals and from 94 immunocompromised individuals (human immunodeficiency virus [HIV]-positive patients and rheumatoid arthritis patients) were collected. JCV DNA was detected by polymerase-chain reaction (PCR) with subsequent nucleotide sequencing of a fragment of the major capsid protein (VP1). JCV was detected in 20.7% of healthy individuals and was found significantly more often in the urine of HIV-positive patients (54.2%; $P < .001$) and rheumatoid arthritis patients (54.4%; $P < .001$). In healthy Irish individuals genotype 1 was the predominant genotype in 62.5%, followed by genotype 4 in 16.7% and genotype 2 in 12.5%. In contrast, genotype 2 was significantly more often isolated from the urine of both HIV-positive patients (60%) and rheumatoid arthritis patients (54.4%; $P < .01$). The pattern of genotype distribution among healthy Irish individuals is in agreement with data reported from other European countries, whereas the overall level of JCV urinary excretion is lower. Previous studies have found genotype 2 significantly more often in cerebrospinal (CSF) samples of patients with progressive multifocal leukoencephalopathy (PML). Here the authors report an increased frequency of genotype 2 in urine samples of immunocompromised non-PML patients. This finding further underlines the hypothesis that there could be biologic differences between JCV genotypes. *Journal of NeuroVirology* (2006) **12,** 39–46.

Keywords: genotypes; immunodeficiency; JC virus; VP1 gene

Introduction

JC virus (JCV) is a human polyomavirus and was isolated for the first time from brain tissue of a Hodgkin's lymphoma patient with progressive multifocal leukoencephalopathy (PML). Serological evidence suggests that JCV infects more than 70% of the human population (Stolt *et al*, 2003). PML is characterized by lytic infection of oligodendrocytes, which causes widespread demyelination in the white matter of the central nervous system (CNS) (Major, 2001). Since its first isolation JCV has been found in brain tissue as well as cerebrospinal fluid (CSF) of PML patients (Major, 2001). Before the acquired immunodeficiency syndrome (AIDS) epidemic, PML was a very rare disease, mainly affecting patients with lymphoproliferative diseases. The advent of AIDS changed the frequency with which this rare CNS disease was observed. PML affects approximately 5% of AIDS patients (Berger, 2003). Other diseases where PML also occurs include autoimmune disorders, such as rheumatoid arthritis, malignancies, or in patients undergoing immunosuppressive therapy

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because of organ transplantation. Recently JCV has received new attention as in patients treated with $\alpha 4$ integrin monoclonal antibody for multiple sclerosis or Crohn's disease, a significant number of PML cases was observed (Kleinschmidt-DeMasters and Tyler, 2005; Van Assche *et al*, 2005).

JCV is thought to persist lifelong in a latent state in the vast majority of humans and may be reactivated under conditions of immune alteration. The main site of persistence is the kidney and JCV urinary isolation rates in healthy individuals vary from 20% up to nearly 50% (Sundsfjord *et al*, 1994; Agostini *et al*, 2001; Pagani *et al*, 2003). Excretion of JCV into the urine increases with age (>30 years) (Agostini *et al*, 2001). Among immunocompromised individuals JCV urinary excretion has been investigated in human immunodeficiency virus (HIV)-positive patients and in patients with autoimmune diseases receiving immunosuppressive treatment (Behzad-Behbahani *et al*, 2004; Tsai *et al*, 1997). Whereas most studies found no increase in JCV urinary excretion in HIV-positive patients in comparison to healthy control subjects, in patients treated for autoimmune disorders the excretion rate increased when patients received more cytotoxic treatments (Sundsfjord *et al*, 1994; Behzad-Behbahani *et al*, 2004; Tsai *et al*, 1997).

The JCV genome is organized into coding regions and the noncoding control region. In contrast to the archetype noncoding control region amplified from urine of healthy and immunocompromised individuals, the noncoding control region of PML brain JCV has been found to have extensive genetic rearrangements.

Genotyping of JCV is performed by sequencing the whole genome, the V-T intergenic region, and a 215-bp segment of the VP1 coding region (between nucleotide positions 1710 and 1924, sequence numbering based on prototype Mad-1; Frisque *et al*, 1984). Comparison of sequence data from the whole genome and the VP1 region suggested that JCV genotyping based on the VP1 fragment is congruent with that defined by the whole genome (Agostini *et al*, 1998b). The JCV genotype classification scheme of Agostini *et al* distinguishes eight genotypes and various subtypes (Agostini *et al*, 2001). Type 1 is found in Europeans, types 2 and 7 in Asians, and types 3 and 6 in Africans. JCV type 8 has been detected in Papua New Guinea and other Pacific islands (Yanagihara *et al*, 2002). Two or more major subtypes within type 1, type 2, and type 3 have been identified (Agostini *et al*, 2001). Type 2, which is found in Asia, is divided into two main subtypes—2A in northeastern Asia and 2B, which is related to the East Asian type and is now found in Europe and the United States. Type 4 has been found in the United States and in Europe and is closely related to genotype 1. Some authors dispute that type 4 is phylogenetically distinct enough from genotype 1 to count as a unique genotype by itself (Hatwell and Sharp, 2000). As VP1 constitutes the receptor binding domain, which is important for viral attachment to the cell, it has been suggested that amino acid variation of VP1 observed in different genotypes could contribute to different pathogenic properties of JCV (Dubois *et al*, 2001). Notably several reports have associated JCV genotype 2 VP1 sequences with PML development in AIDS patients (Agostini *et al*, 1997; 1998a; Ferrante *et al*, 2001).

Here we report for the first time the frequency of JCV urinary excretion in the healthy Irish population. JCV genotype distribution among healthy Irish individuals was analyzed and compared to the genotype distribution in HIV-positive patients and in rheumatoid arthritis patients receiving, among other immunomodulatory drugs, tumor necrosis factor (TNF)-α antibody therapy.

Results

JCV urinary excretion

JCV urinary excretion was investigated by nested polymerase chain reaction (PCR) amplifying a 215-bp fragment of the VP1 major capsid protein gene. Three different cohorts of study subjects were investigated: healthy individuals, HIV-positive patients, and rheumatoid arthritis patients. The sensitivity of the nested PCR was determined by using commercial quantitative JCV standards as five copies of JCV per PCR (data not shown). JCV viruria rate was 20.7% among healthy Irish individuals and was found to be significantly increased in HIV-positive patients (54.2%) and in rheumatoid arthritis patients (54.4%) ($P < .001$; Figure 1).

The average age of the study subjects was 37.8 years among healthy individuals, 36.1 years among HIV-positive patients, and 52 years among rheumatoid arthritis patients. JCV urinary excretion correlated with the age in healthy individuals. It was significantly increased in individuals more than

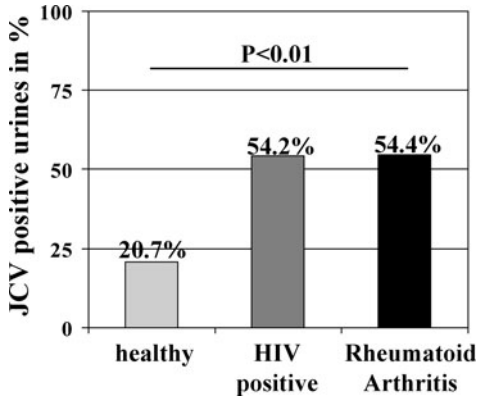

Figure 1 JCV-positive urines in three different patient cohorts. JCV VP1 sequences were amplified from urines of healthy individuals and from urines of HIV-positive and rheumatoid arthritis patients. Healthy individuals 20.7% (25/121), HIV positive patients 54.2% (26/48), and rheumatoid arthritis patients 54.4% (25/46).

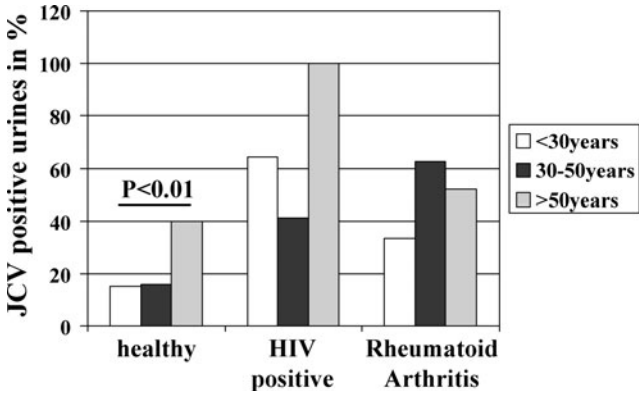

Figure 2 JCV positivity in the urine according to the age of subjects. Healthy individuals, <30 years-15.2% (7/46); 30–50 years-16% (8/50); >50 years-40% (10/25). HIV-positive patients, <30 years-64.3% (9/14); 30–50 years-41.4% (12/29); >50 years-100% (5/5). Rheumatoid arthritis patients, <30 years-33.3% (1/3); 30–50 years-62.5% (10/16); >50 years-51.9% (14/27).

50 years of age ($P < .01$; Figure 2). In contrast there was no significant difference between the urinary isolation rates in the various age groups among HIV-positive or rheumatoid arthritis patients.

JCV genotype distribution in urine

The PCR fragments from urine samples were sequenced and analyzed by BLAST and CLUSTAL/W, comparing the amplified VP1 fragment found in the urine to the eight published JCV genotypes. One sample among healthy individuals and one sample among the rheumatoid arthritis patients showed more than one JCV genotype. By sequencing of the remaining 74 JCV-positive urines we found a total of 15 different strains of JCV (Table 1).

Among healthy individuals genotype 1 amplified as the predominant genotype in 62.5%. Genotype 4 was found in 16.7% of subjects whereas genotype 2 was less frequent with 12.5% occurrence. Genotype 7 was found in 4.2% of healthy samples tested. The African genotype 3 was detected in one of the study individuals (Table 2). No correlation was observed between excretion of the different JCV genotypes and the gender and the age of the subjects.

Among type 1 viruses 80% belonged to subtype 1A and 20% to subtype 1B. The second most prevalent genotype found was genotype 4. One of the genotype 4 strains detected showed a nucleotide substitution at position 1851 (C → G), which has

Table 1 Genotype variants in the VP1 gene fragment

		Nucleotide position														
JCV	*No.*	*1753*	*1771*	*1786*	*1795*	*1804*	*1818*	*1822*	*1836*	*1837*	*1843*	*1849*	*1850*	*1851*	*1869*	*1870*
Type 1A (J02227)	27	A	C	G	A	T	G	G	C	T	G	A	A	C	G	G
DQ 181922	2									**C**						
Type 1B (AF281601)	5	A	C	G	A	T	G	G	C	T	T	A	G	C	G	G
Type 2* (AF015531)	2	A	A	G	A	T	C	G	C	T	T	A	A	C	G	A
Type2 (AF015533)	16	A	A	T	A	T	C	G	C	C	T	A	G	C	G	A
DQ 181923	1										**C**					
Type 2 (AF015536.1)	5	A	A	T	A	T	C	G	C	T	T	A	G	C	G	A
Type 2 (AY536243.1)	4	A	A	G	A	T	C	G	C	T	T	A	G	C	G	A
Type3B (U73501)	2	T	A	G	A	T	C	G	C	T	T	A	A	C	C	G
DQ 181924	1			**T**											**G**	
Type 4 (AF015528)	2	A	C	G	A	T	C	G	C	T	G	A	A	C	C	A
DQ 181925	2										**C**					
AB048565.1	1	A	C	G	A	T	C	G	C	T	G	A	A	G	C	A
Type 7* (AF300965.1)	3	A	A	G	A	T	C	G	C	T	T	A	A	C	G	A
Type 8 (AF396428.l)	1	A	G	G	A	T	C	G	C	T	T	A	A	C	G	A

Note. Nucleotides highlighted in bold are new sequence polymorphisms found in this study and the corresponding gene bank accession numbers are given. Nucleotide numbering is based on Frisque *et al* (1984). As classification of genotype 2 sequences is based on nucleotide variations at only a few positions (nt 1786, 1837 and 1850) and we did not perform whole genome amplification genotype 2 sequences were left un-subclassified.

*In the VPI classification scheme there is no nucleotide difference in typing positions between genotype 2 (AF015531) and genotype 7 (AF300965.1). We found nucleotide differences in nontyping sites between these strains and classification of JCV strains into subtype 2A or 7 was achieved through BLAST search.

Table 2 JCV genotype distribution among healthy Irish individuals and among HIV-positive and rheumatoid arthritis patients

Patients	*Total*	*No positive (%)*	*Genotypes*									
			1A	*1B*	*2[a]*	*2[b]*	*2[c]*	*3B*	*4*	*6*	*7*	*8*
Healthy	121	25** (20.7%)	12	3	—	3	—	1	4	—	1	—
HIV-positive	48	26 (54.2%)	8	—	—	8	4	2	1	—	2	1
Rheumatoid arthritis	46	25** (54.4%)	9	2	2	6	5	—	—	—	—	—

As classification of genotype 2 sequences is based on nucleotide variations at only a few positions (nt 1786, 1837 and 1850) and we did not perform whole genome amplification genotype 2 sequences were left un-subclassified. The amplified genotype 2 sequences were found to align to the following GenBank sequences: 2[a] Alignment by BLAST to AF 015531 (genotype 2A), 2[b] Alignment by BLAST to AF 015533 (genotype 2B), 2[c] Alignment by BLAST to AF 015536.1 or AY 536243.1.
**One sample was a mixed infection with genotypes 1 and 2.

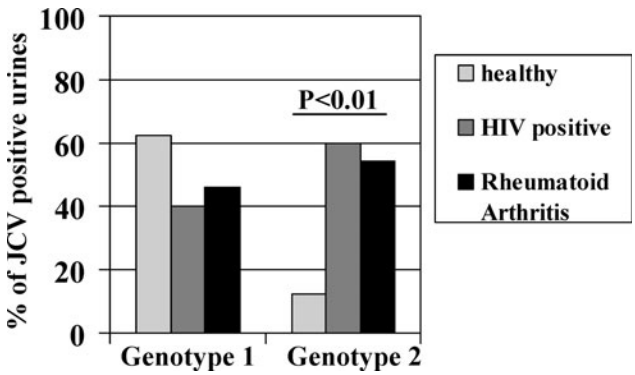

Figure 3 Comparison of JCV genotype 1 and 2 distribution between healthy individuals and HIV-positive and rheumatoid arthritis patients. Only Irish individuals were included in this analysis. The frequency of genotype 1 was healthy individuals 62.5% (15/24), HIV-positive patients 40% (6/15), and rheumatoid arthritis patients 45.8% (11/24). The frequency of genotype 2 was healthy individuals 12.5% (3/24), HIV positive patients 60% (9/15), and rheumatoid arthritis patients 54.2% (13/24).

been previously described as being present in Europe (Agostini *et al*, 2001; Pagani *et al*, 2003). Among the remaining genotype 4 strains we detected an additional nucleotide substitution (G → C) at position 1843 in two strains, which had not been previously described (Table 1). However, this nucleotide substitution was redundant and did not result in an amino acid change. Only one genotype 2 VP1 sequence was found among healthy Irish individuals aligning to GenBank accession number AF 015533 which had been classified on the basis of whole genome sequencing as subtype 2B (Agostini *et al*, 1998c). The single JCV genotype 3 detected was identified as being genotype 3B. There was no difference in the geographic distribution of JCV genotypes in Ireland.

Among immunocompromised individuals (HIV-positive and rheumatoid arthritis patients) there was a significant increase in genotype 2 ($P < .01$) (Figure 3). In HIV-positive patients the four most prevalent genotypes were genotype 2 (46.2%), genotype 1 (30.8%), genotype 7 (8%), and genotype 4(3.8%). The only two genotypes represented in rheumatoid arthritis patients were genotype 2 (54.2%) and genotype 1 (45.8%) (Table 2).

As in healthy individuals the predominant genotype 1 subtype detected within immunocompromised patients was subtype 1 A. Among rheumatoid arthritis patients a new variant of subtype 1A with a nucleotide substitution (T → C) at position 1837 was found in two samples.

In addition to subtype 2B sequences (accession no AF 015533), which were the most prevalent genotype 2 sequence, several other genotype 2 sequences within the VP1 region were detected in immunocompromised individuals. Two samples within the rheumatoid arthritis population were found to align to JCV #226 genotype 2A isolated from the urine of a native American (accession no AF015531). Among the remaining genotype 2 strains, two strains both in the HIV patient cohort and in the rheumatoid arthritis patient cohort were found to be most closely related within the VP1 fragment to JCV SA 27_03 (accession number AY536243.1) reported from a Caucasian leukaemia patient in Africa (Venter *et al*, 2004). This strain had been previously classified subtype 2C, whole-genome amplification and phylogenetic analysis of subtype 2C strains revealed that these fall either into the subtype 2A or 2B category (Agostini *et al*, 1998c). The remaining genotype 2 samples within the HIV and rheumatoid arthritis patients were tentatively identified as genotype 2D (accession number AF015536.1) by their VP1 sequence as described by Agostini *et al* (1998c). As we did not perform whole-genome amplification on these strains, we were unable to unambiguously identify all of the genotype 2 subtypes and therefore left these JCV type 2 strains without subtype classification and grouped them as type 2 (Table 2). Among the genotype 2 strains, a new variant with a nucleotide substitution (T → C) at position 1843 was detected in the urine of a rheumatoid arthritis patient (Table 1). Neither the subtype 1A variant nor the genotype 2 variant gave rise to any amino acid substitutions.

Among African HIV patients two genotype 3 strains were amplified. Both these strains subgrouped into genotype 3B. One of these genotype 3B strains showed two nucleotide substitutions: G → T at position 1786, which had not been described before, and C → G at position 1869. The nucleotide substitution in position 1869 changes alanine in position 134 of the VP1 protein into glycine.

To be able to compare genotype distribution between healthy individuals and HIV-positive and rheumatoid arthritis patients, genotype data were analyzed only on Irish nationals in all study cohorts. Non-Irish HIV-positive individuals were excluded, as their different geographic origin would have influenced the JCV genotype involved. In contrast to

A

JCV		1771	1786	1818	1837	1843	1850	1870
Type 1	Type 1A (J02227)	**C**	G	**G**	T	G	A	**G**
	Type 1B (AF281601)	**C**	G	**G**	T	T	G	**G**
Type 2	Type 2A (AF015531)	**A**	G	**C**	T	T	A	**A**
	Type 2B (AF015533)	**A**	T	**C**	C	T	G	**A**
	Type 2* (AF015536.1)	**A**	T	**C**	T	T	G	**A**
	Type 2* (AY536243.1)	**A**	G	**C**	T	T	G	**A**

B

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                                                117         128
1A (J02226)    94 DLTCGNILMWEAVTLKTEVIGVTSLMNVHSNGQATHD 130
1B (AF281601)  94 DLTCGNILMWEAVTLKTEVIGVTSLMNVHSNGQAAHD 130
2A (AF015531)  94 DLTCGNILMWEAVTLKTEVIGVTTLMNVHSNGQATHD 130
2B (AF015533)  94 DLTCGNILMWEAVTLKTEVIGVTTLMNVHSNGQAAHD 130
2* (AF015536)  94 DLTCGNILMWEAVTLKTEVIGVTTLMNVHSNGQAAHD 130
2* (AY536243)  94 DLTCGNILMWEAVTLKTEVIGVTTLMNVHSNGQAAHD 130
                  **********************:**********:**

1A (J02226)   131 NGAGKPVQGTSFHFFSVGGEAL 152
1B (AF281601) 131 NGAGKPVQGTSFHFFSVGGEAL 152
2A (AF015531) 131 NGAGKPVQGTSFHFFSVGGEAL 152
2B (AF015533) 131 NGAGKPVQGTSFHFFSVGGEAL 152
2* (AF015536) 131 NGAGKPVQGTSFHFFSVGGEAL 152
2* (AY536243) 131 NGAGKPVQGTSFHFFSVGGEAL 152
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Figure 4 Nucleotide and amino acid variations in the amplified VP1 region between JCV genotypes 1 and 2. VP1 gene (nucleotides 1753–1920) and amino acid sequences (94–152) were compared in JCV isolates. (**A**) Nucleotides shown are those at positions were differences were found. Nucleotides common to all genotype 1 or 2 isolates examined in this study are boxed. GenBank accession numbers are given in parentheses for each genotype. Nucleotide numbering is that of Frisque *et al* (1984). (**B**) There was only one genotype-specific amino acid variation (117S →T) within the amplified VP 1 fragment.

healthy individuals, the most frequently encountered JCV genotype between HIV-positive and rheumatoid arthritis patients was genotype 2, with 60% and 54.4%, respectively ($P < .01$) (Figure 3). The vast majority of these genotype 2 viruses aligned to AF 015533 (published as genotype 2 subtype 2B).

Differences in the nucleotide and amino acid sequence in the amplified VP1 region between genotype 1 and genotype 2 are highlighted in Figure 4. Nucleotides at positions 1771, 1818, and 1870 were consistently different between genotypes 1 and 2. The G → C substitution in position 1818 gives rise to an amino acid change: serine to threonine in position 117. In addition to position 117 we observed amino acid differences in position 128, but changes in this site are not genotype specific (Figure 4).

Discussion

The JCV urinary excretion rate was found to be 20.7% among healthy Irish individuals. Worldwide reported JCV urinary isolation rates range from 13% to 50% (Tsai *et al*, 1997; Kitamura *et al*, 2001). Variables influencing isolation rates are the age of the participants and changes in immune status. As observed in other studies the JCV isolation rate increased from 16% in the age group below 50 years to 40% in individuals older than 50 years in this study (Agostini *et al*, 2001).

Several of the previously published studies collected urine samples among hospital inpatients or outpatients. Even though these patients had no obvious diagnosis of immunosuppression, it is possible that other underlying diseases could have impacted on the JCV urinary excretion rate. We collected samples among university staff during regular working hours thereby minimizing possible bias due to impaired health status. The relatively young age of our study population (average age 37.8 years) and the nonhospital setting of this study likely contributed to the lower JCV excretion rate.

The JCV positivity rate in urines of immunocompromised patients was 54.2% for HIV-positive patients (Irish and non-Irish nationality) and 54.4% for rheumatoid arthritis patients. Whereas some studies reported that JCV viruria is not intensified by immunodeficiency caused by HIV infection (Sundsfjord *et al*, 1994; Behzad-Behbahani *et al*, 2004; Ferrante *et al*, 2001), Lednicky *et al* found that JCV excretion in urine was more common in HIV-positive patients but not significantly different from that of the HIV-negative group. Among HIV-infected patients significant differences in JCV shedding were related to $CD4^+$ cell counts (Lednicky *et al*, 2003). Similarly we observed a significant increase of the JCV urinary isolation rate between immunocompetent and HIV-positive individuals (Figure 1). In HIV-positive patients the average $CD4^+$ cell count was lower and the average HIV viral load was higher in patients whose urine was positive for JCV; however, this finding did not reach statistical significance (data not shown).

In rheumatoid arthritis patients it was found that the JCV urinary excretion rate increased with the use of cytotoxic agents compared to noncytotoxic agents (Wang *et al*, 2000). The rheumatoid arthritis patients in our study were treated with TNF-α antibody therapy in conjunction with other immunomodulatory drugs (see list above). The JCV viruria rate among these rheumatoid arthritis patients is strikingly similar to the isolation rate in HIV-positive individuals. TNF-α antibody therapy has been shown to be immunomodulatory and reactivation of tuberculosis has been associated with TNF-α blocking agents (Centers for Disease Control and Prevention [CDC], 2004). Even though there are many reports linking HIV immunodeficiency to an increased risk of developing PML disease, a similar rate of PML disease has not been observed in rheumatoid arthritis patients. The presence of HIV-1 in the brain of infected individuals may directly contribute to the pathogenesis of PML as has been indicated by several *in vitro* studies (Daniel *et al*, 2001; Krachmarov *et al*, 1996).

The genotype distribution in Ireland corresponds to results described in other European countries with genotype 1 being the most frequently encountered genotype.

In contrast to healthy Irish individuals, a different genotype profile was detected in the two cohorts of Irish immunocompromised patients. In both the HIV-positive cohort and in the rheumatoid arthritis cohort the most common genotype was type 2. This is intriguing as a previous comparison of the genotype distribution of JCV amplified from the brain of PML patients with that from urine of non-PML individuals (HIV positive and HIV negative) revealed that genotype 2 strains are more frequently detected in PML brain than in the urinary tract of both the HIV-positive and HIV-negative non-PML control group (Agostini *et al*, 1998a; Ferrante *et al*, 2001). The authors found that infection with genotype 2 is associated with a threefold increased risk of developing PML compared with infection with nontype 2 strains (Agostini *et al*, 1998a). In contrast we report here for the first time a significant difference in the genotype distribution comparing urinary isolates from immunocompetent and immunocompromised individuals.

Although the reasons are unclear it could be speculated that the immunocompromised state may favour the replication of genotype 2. As there is no reason why immunocompromised individuals living in the same geographical area should become infected with a different JCV genotype during their childhood, one would also have to assume that the percentage of the population getting infected with more than one JCV genotype is higher than observed so far and that immunosuppression might allow genotype 2 to replicate more efficiently.

Alternatively, although much less likely, it is possible that the JCV VP1 coding region could undergo sequence changes during persistence in the host as observed for the JCV noncoding control region. Supporting the hypothesis that the coding region could undergo nucleotide changes, Zheng *et al* reported that JCV amplified from PML brain frequently had amino acid substitutions in the VP1 loops. In contrast to results reported in this paper, the authors did not find VP1 amino acid substitutions in urinary isolates of renal transplant patients (Zheng *et al*, 2005). Interestingly whereas only one genotype 2 sequence was identified in healthy individuals we found several other genotype 2 VP1 sequences in HIV positive and rheumatoid arthritis patients. On the basis of existing information, the observed level of nucleotide changes could not cause genotype shift; however, we intend to investigate this by carrying out extensive analysis of quasispecies of VP1 products and whole-genome analysis in future studies.

Overall our finding of significant genotype differences between urinary JCV isolates from healthy and immunocompromised individuals suggests that JCV could undergo genetic changes in the coding region in the pre-PML immunocompromised state. These changes are not restricted to immunodeficiency caused by HIV. As similar nucleotide changes were observed in JCV strains from HIV-associated PML brain (Agostini *et al*, 1998a; Ferrante *et al*, 2001), they might contribute to the disease causing potential of the virus.

Material and methods

Subjects
For the study urine samples from immunocompetent (121) and immunocompromised individuals (94) were collected. Urine samples of healthy individuals were collected among University staff. All the healthy subjects enrolled in the study were Irish and above the age of 18 years. Information regarding sex, age, and the place of residence during the first 18 years of life was also collected. Immunocompromised patient groups included HIV-infected patients and rheumatoid arthritis patients without any clinical evidence of PML from two Dublin teaching hospitals. Additional information collected in HIV-infected individuals was HIV viral load and $CD4^+$ cell counts at the time of specimen collection.

Urine preparation
Experimental protocols were adopted as previously published (Agostini *et al*, 2001; Pagani *et al*, 2003). Samples were stored at 4°C until analysis. Urine (10 to 30 ml) was centrifuged at 4300 rpm for 10 min. The cell pellet was resuspended in phosphate-buffered saline (PBS), recentrifuged, and the supernatant discarded. Viral DNA was then extracted using the Qiagen QIAmp Viral RNA extraction kit according to the manufacturer's protocol.

Amplification protocols
Strict precautionary measures were taken to avoid contamination and each reaction was performed using a negative and a positive control. The negative control contained all the PCR components but not the template; as positive control either JCV containing plasmid or previously identified JCV-positive urine was used. DNA extraction was verified by performing β-actin PCR. Amplified products were loaded on 1.5% agarose gels and visualized through ethidium bromide staining. The sensitivity of the JCV PCR assays was evaluated using quantified JCV standard and comparing visible bands on gel electrophoresis with quantitative data from a JCV lightcycler PCR (see results section).

VP1 amplification
Genotyping of the detected JCV was performed by amplification of a 215-bp fragment of the VP1 gene by nested PCR with TaqDNA polymerase (Promega), using the primers JLP15-5 (5′-CCAGATGAGCATCTTAGGGGTTT-3′) and JLP 16-6 (5′-GATTGCACTGTGGCATTCTTTGG-3′) as outer primers and primers JLP-15 (5′-ACAGTGTGGCCA GAATTCACTACC-3′) and JLP-16 (5′-TAAAGCCTCC CCCCCAACAGAAA-3′) as inner primers. This fragment of the VP1 gene includes the genotyping sites, which can distinguish the eight JCV genotypes and the different subtypes. Amplification was performed using a MJ Research DNA Engine PCR apparatus. The following cycle parameters were used: Denaturation at 95°C for 1 min, annealing in the first round amplification at 57°C for 1 min, annealing in the second round amplification at 63°C for 1 min and extension at 72°C for 1 min, repeated for 35 cycles.

DNA direct sequencing
PCR samples were purified using the High Pure PCR Product Purification Kit (Roche) according to manufacturer's protocol. DNA direct sequencing was then performed using dideoxy nucleotide sequencing with an automated ABI 310 sequencer. For VP1 genotyping primers JLP-15 and JLP-16 (see PCR amplification protocol for sequences) were used as sequencing primers. Sequence homology searches for genotyping were performed using BLAST at National Center for Bioinformatics (NCBI).

Statistical analysis
Statistical analysis was performed by chi-square test.

Reference sequences
GenBank data library accession numbers are given in brackets. JCV type 1 strains [J02227, AF281601], JCV type 2 strains [AF015531, AF015533, AF015536.1, AY536243.1], JCV type 3 strain [U73501], JCV type 4 strains [AF015528, AB048565.1], JCV type 6 [AF015537], JCV type 7 strain [AF300965.1], JCV type 8 strain [AF396428.1]. GenBank accession numbers of JCV sequence polymorphisms described in this study are DQ 181922, DQ 181923, DQ 181924, and DQ 181925.

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