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Identical rearranged forms of JC polyomavirus transcriptional control region in plasma and cerebrospinal fluid of acquired immunodeficiency syndrome patients with progressive multifocal leukoencephalopathy

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Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease of the central nervous system (CNS) caused by the human polyomavirus JC (JCV). JCV has a hypervariable noncoding transcriptional control region (TCR) that spans the origin of replication of the JCV genome through to the first ATG start codon for late gene transcription. The archetype form of TCR is frequently found in the urine and kidneys of healthy and immunocompromised subjects. However the rearranged forms, whose prototype is Mad-1, possibly generated by deletion and duplication of segments of the archetype sequence, are found in the brain and cerebrospinal fluid (CSF) of PML patients. In this study the authors compared JCV TCR detected in paired CSF, plasma, and urine samples of 11 acquired immunodeficiency syndrome (AIDS) patients affected by PML to try to determine where the rearranged JCV TCRs are selected. In one patient, it was also possible to amplify and sequence the TCR in the brain and lymphocytes. Moreover, in 5/11 patients, the CSF, plasma, and urine samples corresponding to 2 months after PML development were available; and in another patient, it was possible to sequence the TCR in plasma and lymphocytes sampled 8 months before the onset of PML. The presence of the same TCR sequences in all the CSF and plasma samples taken from individual patients could strengthen the hypothesis that the blood is a compartment where JCV may replicate and undergo rearrangement of the TCR. This further supports the hypothesis that JCV reaches the brain by a hematogenous route and indicates that the JCV TCR sequences detected in plasma could be used as an early marker of JCV pathogenicity before the clinical appearance of PML in immunocompromised patients. *Journal of NeuroVirology* (2003) **9,** 551–558.

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Introduction

The human polyomavirus JC (JCV) is a small DNA virus that infects a large majority of the world population. Asymptomatic JCV infection occurs during childhood, with seroprevalence in about 80% of the adult population (Padgett and Walker, 1973). After primary infection, JCV establishes latent infection in the kidneys and probably in the bone marrow, the hematopoietic system, the leukocytes, and the

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central nervous system (CNS) (Andreoletti *et al*, 2002; Chester *et al*, 1983; Dörries *et al*, 1994; Elsner and Dörries, 1992; White *et al*, 1992). Immunodeficiency may determine the reactivation of the virus and its urinary excretion. In severely immunocompromised patients, JCV is the etiological agent of progressive multifocal leukoencephalopathy (PML) (Henson *et al*, 1991; Telenti *et al*, 1990; Tornatore *et al*, 1992; Weber and Mayor, 1997), a fatal CNS demyelinating disease.

The importance of PML has been stressed widely in the past decade, particularly following the appearance of the acquired immunodeficiency syndrome (AIDS). Other conditions associated with PML include renal and bone marrow transplantation (Yogo *et al*, 1991; Re *et al*, 1999), lymphoproliferative diseases (Gallia *et al*, 1997), and leukemia (Hammarin *et al*, 1996). In patients latently infected by JCV, it has been suggested that, in addition to severe immunodeficiency, other factors are needed for PML development. Indeed, in recent years, several studies have focused on the hypervariable noncoding transcriptional control region (TCR) structure that spans the JCV replication origin genome through to the first ATG start codon for late gene transcription (Ault and Stoner, 1993; Agostini *et al*, 1997; Caldarelli-Stefano *et al*, 1999; Ciappi *et al*, 1999; Pfister *et al*, 2001; Raj and Khalili, 1995; Vaz *et al*, 2000; Yogo *et al*, 1990).

Urinary JCV strains have a nonrearranged TCR named archetype sequence (Yogo *et al*, 1990). JCV strains recovered from the brain and cerebrospinal fluid (CSF) of PML patients contain rearranged forms of JCV TCR, the prototype of which is Mad-1 (Walker and Frisque, 1986). These rearranged forms may be generated by deletions and duplications of the archetype sequence (Agostini *et al*, 1996; Iida *et al*, 1993; Yogo *et al*, 1990). Mad-1 TCR is characterized by two 98-bp tandem repeats that feature a TATA box and also by the absence of the 23- and 66-bp fragments that are present in the archetype form (Figure 1). It has been suggested that the archetype form circulates among the population and that rearrangements occur within the host. It has also been shown that rearranged JCV replicates more efficiently than archetype JCV in cultured cells (Daniel *et al*, 1996). Furthermore, recent reports (Caldarelli-Stefano *et al*, 1999; Ciappi *et al*, 1999; Jensen and Major, 2001; Newman and Frisque, 1999; Pfister *et al*, 2001) have suggested that the variability of JCV TCR may help trace the pathway of JCV from the site of initial infection to the target cells as PML progresses. However, both the variability of the TCR in different anatomic compartments and the relationship between JCV TCR rearrangement and the outcome of PML remain unclear.

Herein we compared the JCV TCR sequences detected from paired CSF, plasma, and urine samples of 11 AIDS patients affected by PML. We sought to evaluate in which compartment the rearranged forms originate and whether JCV could reach the brain via a hematogenous route. We also assessed whether the JCV TCR structure changes as PML progresses. This knowledge may help clarify one aspect of the pathogenesis of PML and could provide a useful prognostic virological marker.

Results

We analyzed the JCV TCR sequences detected from paired CSF, plasma, and urine samples of 11 PML AIDS patients (Table 1). In all samples, except in two plasma samples from the same patient, JCV DNA was detected.

Our analysis of the JCV TCR detected in the paired CSF and plasma of each patient showed the same rearranged form of JCV TCR in both samples (Figure 2). In patient 2, we detected the same rearranged form of JCV TCR in the plasma, lymphocytes, CSF, and brain.

In 10/11 patients we found the tandem repeat elements; only one patient (patient 8) featured the same archetype-like form of JCV TCR in his plasma and CSF samples, characterized by a 6-bp deletion in region D.

Moreover, in 5/11 patients the CSF, plasma, and urine samples corresponding to 2 months after the onset of PML were available for amplification and

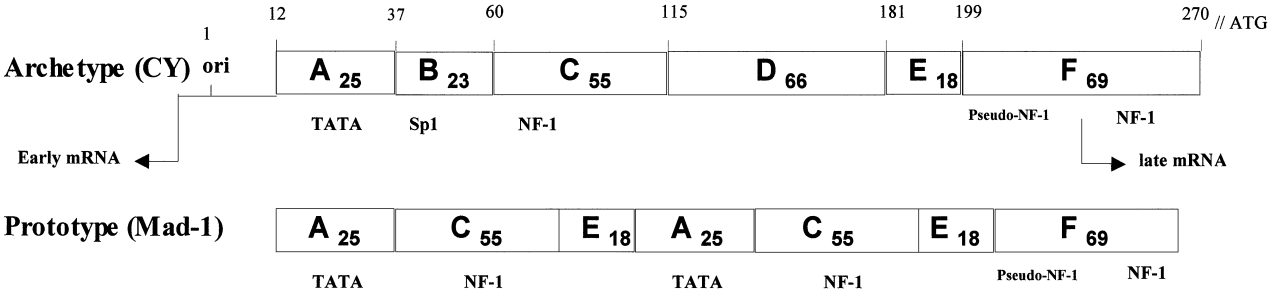

Figure 1 Schematic representation and comparison between the TCR of JCV archetype CY (Yogo *et al*, 1990) and JCV prototype Mad-1 (Frisque *et al*, 1984). ORI indicates the origin of replication (at nucleotide 1 on genome). The TATA box and the nuclear factor 1 are represented by TATA and NF-1, respectively. Sp-1 is the binding site for the transcription factor Sp-1. Regions A to F and their size are indicated in the boxes according to Ault and Stoner (1993).

Table 1 Patients and clinical parameters

Patient	*MRI findings*	*Outcome*	*CD4 cell count/μl*	*HIV RNA corresponding to PML development*	*Anti-HIV therapy*	*Therapy after PML diagnosis*
1	Single hipointense T_1 lesion in periventricular white matter of the right lobe temporal	Stabilization and improvement	132	1.7 $\log_{10}$	AZT + 3TC + RTV + IDV	Cidofovir
2	Multiple hipointense T_1 lesions in left parietal lobe and centrum semiovale	Deceased	60	4.3 $\log_{10}$	No therapy	No therapy
3	Multiple hipointense T_1 lesions in the left frontal and parietal lobes	Progression and deceased	69	3.7 $\log_{10}$	3TC + AZT + NFV	Cidofovir
4	Multiple and bilateral hipointense T_1 lesions in the right parietal and frontal lobes and in the left periventricular and subcortical white matter	Progression	137	4.8 $\log_{10}$	IDV + RTV + D4T + DDI	Cidofovir
5	Multiple hipointense T_1 lesions in the right frontal and temporal lobes	Progression and deceased	94	4.6 $\log_{10}$	AZT + 3TC + IDV	Cidofovir
6	Multiple hipointense T_1 periventricular lesions and in right frontal and parietal lobes	Progression and deceased	22	5.7 $\log_{10}$	No therapy	No therapy
7	Lesions hipointense in T_1 in right occipital lobe and in subcortical colossus corpus	Progression and deceased	11	5.6 $\log_{10}$	3TC + IDV + ABC	Cidofovir
8	Lesions hipointense in T_1 in the left frontal and parietal lobes and in right temporal lobe	Progression	74	4.9 $\log_{10}$	3TC + IDV + D4T	Cidofovir
9	Lesions periventricular in frontal and temporal lobes	Stabilization	117	2.6 $\log_{10}$	D4T + ·3TC + RTV + IDV	Cidofovir
10	Multiple lesions hipointense T_1 in left temporal lobe	Stabilization	69	Not determined	No therapy	Cidofovir
11	Single lesion in cerebellum	Progression	33	Not determined	D4T + DDI + effavirez	Cidofovir

Abbreviations: AZT, zidovudine; 3TC, lamivudine; D4T, stavudine; DDI, didanosine; IDV, indinavir; RTV, ritonavir.

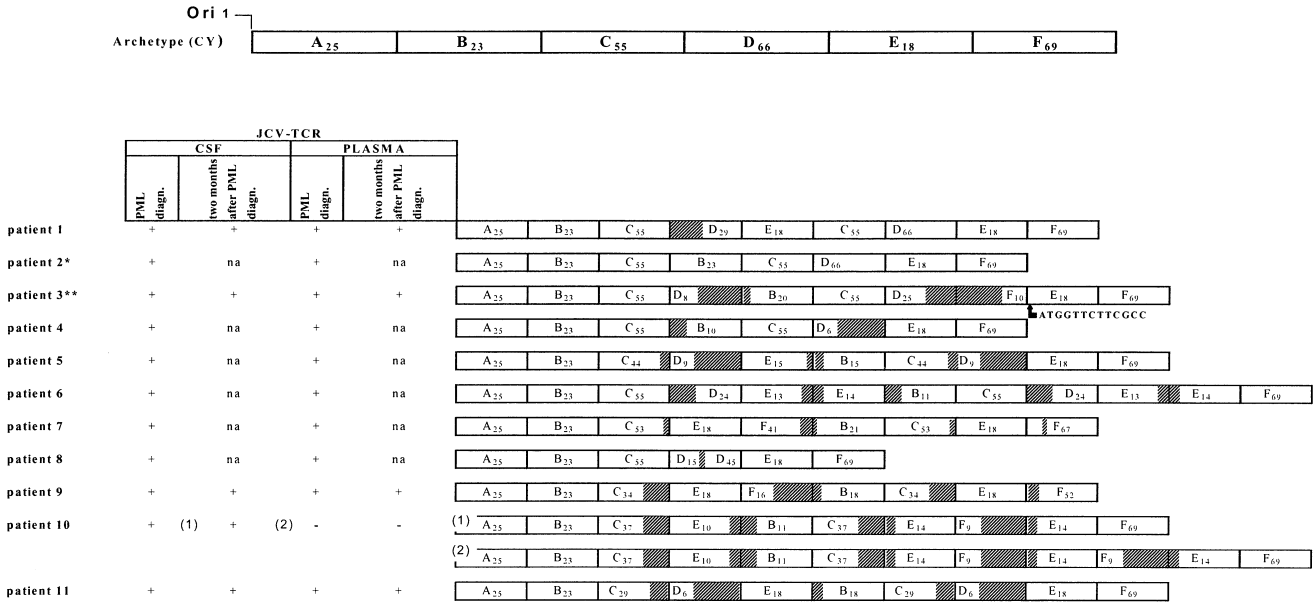

Figure 2 Sequencing results of the JCV TCR from CSF and plasma of AIDS patients with PML. The CY archetype form of TCR (Yogo *et al*, 1990) is shown at the top. Regions A to F and their respective sizes are represented and numbered in the box (Ault and Stoner, 1993; Agostini *et al*, 1997). For the samples, the shading in the boxes indicates the deletions and the arrow represents the insertion; na, not available. *In patient 2, brain and lymphocytes samples that showed the same rearranged form found in his plasma and CSF were also sequenced. **In patient 3, the plasma and lymphocyte specimens corresponding to 8 months before onset of PML were available for amplifying and sequencing; both samples featured the identical JCV TCR form detected in CSF and plasma at the onset and 2 months after PML. [(1), (2)]In patient 10, different but related forms of JCV TCR in different CSF samples were detected.

sequencing. Four of them featured identical JCV TCR found in their CSF and plasma at the onset of PML. In just one case (patient 10), JCV DNA was not detected in the two plasma samples—it was found only in the CSF samples, which showed related, but different, JCV TCRs (Figure 2).

In patient 3, we were able to analyze plasma and lymphocyte specimens sampled 8 months before the onset of PML. These showed a JCV TCR rearrangement identical to the one observed in paired CSF and plasma samples at the onset of PML.

All the urine specimens showed the archetype or archetype-like forms of JCV TCR. The urine samples of patient 1 showed a 13-bp deletion between regions B and C. Urine samples obtained from patient 3 at the onset of PML and 2 months later showed different JCV TCRs. The first urine sample featured an archetype JCV TCR, whereas the second one showed a deletion of the first 13 nucleotides in region F (Figure 3).

Discussion

In the present study, we evaluated the JCV TCR sequences detected in CSF, plasma, and urine samples from PML AIDS patients, in order to clarify how JCV spreads within the host, to determine where the rearranged JCV TCR is selected and to learn more about the role of JCV TCR rearrangement in PML. Several authors have sequenced the JCV TCR region in different anatomic compartments. However, our study furthermore analyses prospectively the hypervariability of TCR in clinical samples of a considerable number of AIDS patients with PML. Our analysis of paired

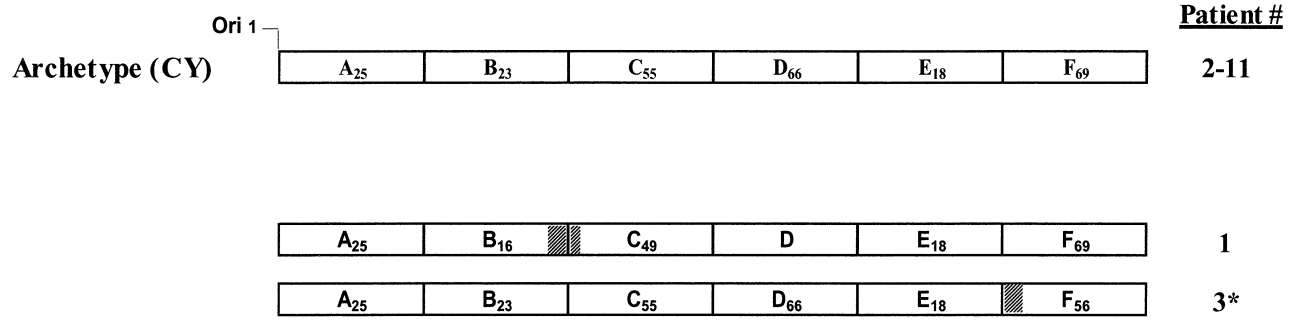

Figure 3 Sequencing results of JCV TCR from urine specimens. The CY archetype form of TCR (Yogo *et al*, 1990) is shown at the top. For patients 1 and 3*, the shading in the boxes indicates the deletions.

CSF and plasma samples from 11 AIDS patients affected by PML showed rearranged JCV TCR forms in 10/11 cases (Figure 2). By contrast, only one patient featured the archetype-like forms of JCV TCR in his CSF and plasma samples.

Like other authors (Ciappi *et al*, 1999; Pfister *et al*, 2001), we detected rearranged JCV TCR forms in CSF and in plasma samples. We furthermore observed identical JCV TCR sequences in the paired CSF and plasma samples from each patient. This strongly suggests that JCV is not present in the CNS latently but appears only after reactivation. The JCV TCR rearrangements could be selected in the blood and JCV may reach the brain via a hematogenous route. In addition, we detected JCV DNA in a very high percentage of plasma samples of PML patients. This suggests that JCV reactivation could take place in this compartment at the time of PML development and that productive JCV infection could be compartmentalized here. Monitoring of JCV TCR in the blood could therefore be used as an early marker of JCV virulence before the clinical appearance of PML in immunocompromised patients. However, the question of in which compartment (plasma or lymphocytes) JCV DNA is more easily detected remains unanswered. Previous reports indicated that peripheral blood lymphocytes could constitute a site of JCV latency after primary infection (Dubois *et al*, 1997) and that JCV adheres to surface receptors of blood cells (Wei *et al*, 2000). In our experience, plasma samples appear to be a more suitable specimen for detecting JCV DNA. Research should be focused on which blood cell type is involved in transporting JCV to the brain and crossing the blood-brain barrier.

Our analysis of the JCV TCR region in patient 3 deserves special mention, as it reinforces our previous hypotheses. We found the same rearranged JCV TCR form observed in paired CSF and plasma samples corresponding to the onset of PML, 2 months later in plasma, and in lymphocytes specimens sampled 8 months before PML occurred. If subsequently confirmed in a larger number of patients, the presence in plasma or lymphocytes of rearranged TCR many months before PML appears may be a useful marker in the clinical management of PML and could allow us to identify possible candidates for developing PML.

It is not clear how JCV enters the CNS or how the lesions caused by JCV expand in the CNS. Several studies suggest that variability in the JCV TCR region may help trace the pathway of JCV from the site of initial infection to the target cells; that rearranged forms first originate in plasma; and that further rearrangement may occur once the JCV invades the CNS. Therefore, in this study, we analyzed the JCV TCR region from plasma and CSF samples corresponding to 2 months after the diagnosis of PML in 5/11 patients. In 4/5 patients, the same JCV TCR form found in plasma and CSF at the time of onset of PML was detected in both samples. In just one case (patient 10), JCV TCR was not detected in the two plasma samples—it was only found in the CSF samples, which showed related, but different JCV TCRs (Figure 2). In contrast to a recent study (Pfister *et al*, 2001), when we analyzed the JCV TCR in five different compartments of patient 2, we found the archetype TCR form in the urine sample and the same rearranged form of JCV TCR in the plasma, lymphocytes, CSF, and brain. Our results support the idea that rearrangements of JCV TCR are a cause and not a consequence of PML, and that the conversion from archetype to rearranged forms occurs when JCV goes from the urine to the blood. They also suggest that the rearrangement of JCV TCR rarely modifies its structure in the CNS during PML progression.

Our analysis of CSF and plasma samples taken from 10/11 AIDS patients affected by PML (Figure 2) showed JCV TCR rearranged forms with tandem repeat elements. The presence of the 23- and 66-bp insertions (although the latter with partial or complete deletion) and only one TATA box suggests that the substrate for this rearrangements is an archetype regulatory region. By contrast, only one patient featured the archetype-like forms of JCV TCR in his CSF and plasma samples, characterized by a 9-bp deletion in region D. Archetype JCV TCR, although rare, has been recently described in CSF samples of PML AIDS patients (Pfister *et al*, 2001), supporting the hypothesis that archetype JCV is not restricted to the urinary tract but may replicate in glial cells. Even so, further attention needs to be given to the factors restricting the lytic activity of the archetype form in primary human glial cells.

In comparison to the most recent classification scheme for the JCV TCR variant (Jensen and Major, 2001), we detected mainly the II-R structure in CSF and plasma samples sequenced. II-R, generally defined as PML type, is characterized by sequence section repeats and deletion and by the insertion of regions B and D, although with some degree of deletions. In contrast to other studies (Vaz *et al*, 2000), the I-R structure, which is indicative of the prototype MAD-1 sequence and other related forms, was not observed. This excludes the possibility of contamination during amplification, because we used the plasmid pM1TC containing JCV Mad-1 as a positive control in our experiments.

Furthermore, an archetype JCV TCR form was observed in all urine specimens, except those of two patients, previously described (Figure 3), which showed the archetype-like form of JCV TCR. We did not find a tandem repeat element in urine specimens. This indicates that, although unusual minor changes in the urinary tract can occur, the length of rearrangement rarely exceeds 10 to 15 nucleotides.

In accordance with a recent report (Sala *et al*, 2001), and in contrast to another study (Pfister *et al*, 2001), our analysis of JCV TCR sequences from these 11 AIDS-related PML patients did not show a correlation between rearranged forms of JCV TCR and the

progression of PML. The JCV TCR form was specific to each patient and independent of the clinical outcome. Contrary to a recent study (Ferrante *et al*, 2002), in one patient (patient 8), who died 4 months after PML diagnosis, we found an archetype-like form of JCV TCR. However, three patients (patients 1, 9, and 10) with a favorable outcome featured rearranged forms of JCV TCR.

Our sequence analysis of JCV TCR rearrangements from plasma and CSF samples revealed that regions A, B, and C were invariably present. Sequence sections proximal to the origin of replication are the most conserved. Duplications mainly occur in regions B, C, and E. Region D was generally partially or completely deleted; in only two cases was region D fully retained. Region F was always entirely retained, except in the CSF and plasma samples of patient 9 and in the second urine specimens of patient 3, which showed the deletion of the first 17-bp and the first 13-bp nucleotides, respectively. This deletion, which affected the pseudo NF1 motif, did not prove to be relevant to JCV replication. Besides, region F is often incorporated between groups of repeat elements. In only one case (patient 3) did we detect a 13-bp insertion corresponding to another portion of the JCV genome located downstream of region F.

The distribution of changes from regions A to F indicates that regions A, B, and C play a critical role in the replication of JCV in CNS (Ault and Stoner, 1993; Ciappi *et al*, 1999; Raj and Khalili, 1995). Indeed, region A contains the TATA BOX, which appears to be crucial for early transcription. Furthermore, overlapping the TATA box, the oct 6/ts1 binding site is present. oct 6/ts1, interacting in synergy with the amino-terminal region of the large T antigen, enhances viral DNA replication. Another key factor in brain-specific JCV early transcription is the NF-1 motif, located at the beginning of region C. Its importance is evidenced by its frequency of appearance. Also, region B was retained in all JCV TCR sequences, indicating that the Sp1 motif, contained in this region, is involved in the regulation of glial-specific gene expression, together with the NF-1 motif. In all sequences the pentanucleotide sequence, an activator of early JCV promoter, is divided into two parts located downstream of the TATA box (5′-AGGGAAGG-3′) and at the beginning of region C (5′-GA-3′) respectively.

We observed a rearranged JCV TCR form with tandem repeat elements in most plasma and CSF samples of PML-affected patients. All patients individually showed the identical JCV TCR form in their CSF and plasma samples, suggesting a viral reactivation in the latter compartment. JCV TCR monitoring in immunocompromized patients may allow JCV reactivation to be detected at an early stage. By enabling a more appropriate therapeutic approach to be taken, this could help prevent the fatal progression of the disease, still evident in the highly active antiretroviral therapy (HAART) era. Many questions on the hypervariability of JCV TCR region and PML pathogenesis are still unclear. For instance, dividing the six blocks into shorter windows should enable a deeper analysis to be performed of the many sequences. This could help us elucidate crucial events as PML progresses and understand how the sequence modification of the promoter/enhancer structure can alter the progression of PML. In addition, novel molecular methods could be used to detect new cellular determinants involved in the modulation of the cellular specificity of JCV and in the production of myelin basic protein.

Materials and methods

Clinical samples and DNA extraction

The study was performed on paired CSF, plasma, and urine samples of 11 PML AIDS patients (Table 1). In five of them, the CSF, plasma, and urine samples corresponding to 2 months after the onset of PML were available for amplifying and sequencing. In only one patient was it possible to sequence JCV TCR in five different compartments (CSF, brain, lymphocytes, plasma, and urine). Also, in one patient, we were able to analyze plasma and lymphocyte samples corresponding to 8 months before the onset of PML. Viral DNA from clinical samples was extracted as previously described (Casas *et al*, 1995). Briefly a total of 100 μl of clinical specimens was incubated for 10 min at room temperature with 400 μl of a guanidinium thiocyanate lysis buffer. The DNA was extracted by isopropanol/ethanol precipitation and pellet was dissolved in 5 μl of sterile double-distilled water. The lymphocyte samples were separated in the laboratory of origin using a Fycoll procedure and sent to our center for DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing.

Amplification of JCV TCR by nested PCR

Clinical specimens were screened for JCV DNA presence by using a qualitative multiplex nested PCR for JC, BK, and SV40 polyomaviruses (Fedele *et al*, 1999).

The TCR region was amplified by nested PCR (nPCR) in which ORI 1 (5′ GATGAGCAACTTTTACACCTTG 3′) (nucleotides 4833 to 4854 of JCV Mad-1) and ORI 2 (5′ CTTACCTATGTAGCTTTTGG 3′) (nucleotides 526 to 507 of JCV Mad-1) were used as sense and antisense primers, respectively, for the first round. For the nested reaction, we used the sense primer ORI 3 (5′ GAAAAACAAGGGAATTTCCCTGGCC 3′) (nucleotides 5081 to 5105 of JCV Mad-1) and the antisense primer ORI 4 (5′ TGAGCTCTTTTTTTAGTTCCACTCC 3′ of JCV Mad-1) (nucleotides 382 to 358). Nucleotide sequences for complete genome of JCV (accession numbers AF015526, AF015527, AF015528, AF015531, AF015533, AF015536, AF015537, J02226, U73501, U73502, U73500, U61771, AF004340, AF004349,

AF004350) were obtained from Gene Bank databases. The primers were selected on conserved sequences using the Hint-PCR program (Dopazo and Sobrino, 1992).

The first round was performed in 50 μl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 20 pmol of each outer primers, 2.5 U of Taq DNA polymerase, and 5 μl of extracted DNA. Amplification was carried out in PTC-200 Peltier Thermal Cycler (MJ Research, MA, USA). After an initial denaturation step of 2 min at 94°C, 40 cycles consisting of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s were performed; a final extension step at 72°C for 5 min was conducted. The second round was carried out by adding 1 μl of first round to 49 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 100 μM dNTP, 10 pmol of each outer primers, and 1.25 U of Taq DNA polymerase. The thermocycler parameters setting were the same used in the first round, except that annealing temperature was 65°C; a total of 30 cycles were used for complete the nPCR. Each assay of genomic amplification included water as negative control and plasmid pM1TC containing JCV Mad-1 as positive control. The size of the fragment that was amplified from a JCV Mad-1 DNA was 401 bp.

Direct nucleotide sequencing

Sequencing of the JCV TCR was performed directly after amplification of the nPCR products (Ciappi *et al*, 1999; Dörries *et al*, 1998). Briefly, the samples after nPCR were purified by using QIAquick spin columns (Qiagen GmbH, Germany), as described by the manufacturer.

Both strands were sequenced with primers ORI 3 and ORI 4, which were used as inner primers in the nPCR previously described. The sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer.

Sequence analysis of amplified products

All the sequences were first analyzed with ABI Prism 3700 DNA Analyzer (Applied Biosystem, Foster City, CA, USA). The consensus sequences were aligned either with the CY archetype sequence (Yogo *et al*, 1990) and arbitrarily divided into A, B, C, D, E, and F blocks consisting of 25 bp, 23 bp, 55 bp, 66 bp, 18 bp, and 69 bp, respectively (Ault and Stoner, 1993), in order to highlight their similarity with the archetype or their rearranged form. The program MACAW 1.01 generated the alignment of consensus sequences.

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