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Detection and typing of JC virus in autopsy brains and extraneural organs of AIDS patients and non-immunocompromised individuals

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The distribution of JC virus (JCV) variants in the brain, lung, liver, kidney, spleen and lymph nodes collected at autopsy from AIDS patients with (Group A: 10 Ss) and without (Group B: 5 Ss) progressive multifocal leukoencephalopathy (PML) and from HIV-negative patients (Group C: 5 Ss), was examined by amplifying the JCV large T antigen (LT), the regulatory (R) and the VP1 regions. Among the samples from the PML patients, JCV DNA was detected in all of the demyelinating areas, in 60% of the lesion-free brain tissues, in 60% of the lung tissues and in 40% of the spleen and kidney tissues, whereas all liver and lymph node sections were negative. JCV DNA was also found in two of the five brain specimens, in two of the five kidney specimens, in one of the five lung specimens from the HIV-positive patients without PML and in the brain specimens from two of the five HIV-negative subjects. Nucleotide sequence analysis indicated that all of the R region amplified from extraneural tissues had rearrangements similar to those of the Mad-4 strain and that VP1-region amplified products were similar to the Mad-1 strain. In the brain specimens from two PML patients, we found a unique rearranged R region, along with a VP1 region of JCV type 2. In addition, an almost unique variant with multiple rearrangements in the R region and unusual base mutations in the VP1 region was detected in the brain sample from another PML patient. The data indicate that diffuse visceral involvement of JCV is particularly frequent in AIDS patients with PML. Moreover, the presence of rearrangements and mutations, involving different regions of the viral genome, observed in PML-affected brain tissues, could represent a risk factor for the development of PML in immunosuppressed individuals.

Keywords: PML; PCR; nucleotide sequence; JCV regulatory region; JCV genotypes; extraneural tissues

Introduction

Progressive multifocal leukoencephalopathy (PML) is a human demyelinating disease of the central nervous system (CNS), caused by a lytic infection of oligodendrocytes by the JC virus (JCV). JCV is a polyomavirus which after primary infection, involving a large majority of the population worldwide during childhood, establishes a persistent infection in the kidney (Chesters *et al*, 1983; Shah, 1996). JCV is frequently reactivated with production of viral particles and viruria, without presenting any apparent clinical symptoms. In immunosuppressed subjects, however, JCV reaches the brain, probably carried by peripheral blood cells, and causes PML (Major *et al*, 1992; Atwood *et al*, 1992; Shah, 1996).

Although JCV infection is widespread, as indicated by the detection of specific serum antibodies in about 80% of the adult population, the frequency of PML is low even among patients affected by acquired immunodeficiency syndrome (AIDS). It has therefore been suggested that in addition to immunosuppression and the presence of JCV, other factors are needed for the development of PML (Padgett and Walker, 1983; Major and Ault, 1995).

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In this regard, in recent years attention has focused on the possible roles of viral rearrangements and JCV subtypes in the pathogenesis of PML.

The JCV circular genome is divided into three functional regions: the early region, encoding the regulatory proteins, the large T (LT) and the small t antigens; the late region encoding the structural proteins VP1, VP2 and VP3 and the agnoprotein; the noncoding regulatory (R) region containing the DNA replication origin and variable promoter-enhancer sequences. The R region controls viral DNA replication from the replication origin and early and late gene transcription from a bi-directional promoter. The R region can show deletions and duplications, which could alter viral DNA replication and modify the capability to replicate in glial cells (Loeber and Dorries, 1988; Krebs *et al*, 1995; Zoltick *et al*, 1995). The analysis of the R region can identify an archetypal JCV, as has been commonly detected in the urine of healthy and non-immunosuppressed individuals, as well as re-arranged types, which have often been isolated from brain tissue specimens from subjects with PML, and thus defined as PML-type regulatory sequences (Loeber and Dorries, 1988; Yogo *et al*, 1990; Iida *et al*, 1993; Ault and Stoner, 1993).

Genomic variations are not limited to the R region, but can also be found within the VP1 and the V-T intergenic regions (Yogo *et al*, 1990; Ault and Stoner, 1992; Iida *et al*, 1993; Zoltick *et al*, 1995; Agostini *et al*, 1995, Guo *et al*, 1996; Agostini *et al*, 1998). Sequence analyses of these regions have made it possible to define various JCV genotypes. Type 1, which was first isolated from brain tissue from a North-American PML patient, type 2, which was isolated from a European, and type 3, found in African subjects (Loeber and Dorries, 1988; Guo *et al*, 1996; Agostini *et al*, 1995, 1996b).

PML is usually diagnosed by means of the detection of JCV DNA in brain tissue or in cerebrospinal fluid (CSF) samples using polymerase chain reaction (PCR) (Arthur *et al*, 1989; Telenti *et al*, 1990; Brouqui *et al*, 1992; Moret *et al*, 1993; Gibson *et al*, 1993; Bogdanovic *et al*, 1994; Weber *et al*, 1994; Fong *et al*, 1995; Cinque *et al*, 1996; Hammarin *et al*, 1996; Ferrante *et al*, 1997). Due to its high sensitivity, PCR has recently seen widespread employment to verify the presence of JCV DNA in samples from other organs besides the brain, from both PML and non-PML patients (Chester *et al*, 1983; Houff *et al*, 1988; Elsner and Dorries, 1992; Ault and Stoner, 1994; Schatzl *et al*, 1994; Ferrante *et al*, 1995; Vago *et al*, 1996).

In order to evaluate the distribution of JCV in various organs, we examined brain, lung, lymph node, spleen, liver and kidney tissue sections collected at autopsy from a total of 20 Subjects (Ss). These subjects included AIDS patients with (Group A: 10 Ss) and without (Group B: 5 Ss) PML, and a group of HIV-negative subjects (Group C: 5 Ss), whose deaths were attributable to diseases unrelated to AIDS.

An outer long PCR (L-PCR) was performed for each sample, followed by three different inner amplifications for each region of the JCV genome: early LT, late VP1, and the non-coding R region. Moreover, VP1- and R-region PCR products were analysed by direct automated sequencing to investigate possible relationships between specific JCV types and PML.

Results

Distribution of JCV DNA

The distribution of JCV LT DNA among the three patient groups is reported in Table 2, subdivided according to the various tissue specimens (excluding the lymph node specimens). It is possible to note from this table that all ten brain tissue sections collected from demyelinating lesions were positive for JCV LT DNA in the PML group (Group A), while the frequency of JCV DNA was lower for the brain tissue sections without demyelination (60%) and for the lung (60%), spleen (40%), and kidney (40%) samples. The PCR yielded negative results for all liver samples, the three lymph node samples available from Group A, all three lymph node samples available

Table 1 JCV primer positions and sequences used in PCR and in direct sequencing reactions

Region		*Position (*)*	*Sequence*	*Size (bp)*
Outer				
	JRE1	4989–5009	5′-CCT CCC TAT TCA GCA CTT TGT-3′	
Long	JC2	4573–4592	5′-GCT TCA GAC AAT GGT TTG GG-3′	4734
Inner				
	PEP1	4255–4274	5′-AGT CTT TAG GGT CTT CTA CC-3′	
LT	PEP2	4408–4427	5′-GGT GCC AAC CTA TGG AAC AG-3′	173
	JRI1	5087–5107	5′-CTC CAC GCC CTT ACT ACT TCT-3′	
R	JRE2	281–301	5′-TAC GTG ACA GCT GGC GAA GAA-3′	313
	VPN3	2107–2126	5′-TTT TGG GAC ACT AAC AGG AG-3′	
VP1	VPN4	2481–2500	5′-GTC AAC GTA TCT CAT CAT GT-3′	394

*Sequence numbering is based on prototype Mad-1 (Frisque *et al*, 1984).

from Group B and the only one available from Group C.

Among the HIV-positive subjects without PML (Group B), JCV DNA was also found in the non-PML-affected brain tissue sections from two (40%) patients, in two (40%) kidney specimen and in only one (20%) lung tissue specimen.

All the extraneural tissue specimens from Group C were negative, while JCV LT DNA was detected in the brain tissue samples from two (40%) of these HIV-negative subjects. Among the PML group, the majority (80%) were found to have at least one extraneural tissue sample positive for JCV LT DNA, in addition to the brain sections, whereas in two cases viral DNA was detected only in the brain sections showing demyelinating lesions.

Regulatory region sequence analysis

As reported in Table 3, PCR sensitivity was lower in the case of the amplification of the R region, compared to that of the LT region. In fact, for Group A, JCV R-region DNA was amplified in seven brain sections from areas with demyelinating lesions and in three lesion-free sections, as well as in four lung, one kidney and two spleen sections. The R region was also amplified in two brain sections, one lung and one kidney section from Group B, but in none of the samples from Group C.

A total of 21 R-region amplified products were thus available for direct sequencing. Computer-assisted analysis of all the samples obtained from all the sections without demyelinating lesions and from the lung, spleen, and kidney revealed a close relationship of the isolates with the Mad-1 strain. However, these amplified products had a 19-bp deletion of the second TATA box, which is typical of the Mad-4 strain (Martin *et al*, 1985).

In Group A, the seven PML-affected brain tissue sections with demyelinating lesions showed greater variability of the nucleotide sequences of the amplified products. A schematic comparison of these sequences with the archetype and Mad-1 sequence can be found in Figure 1. As one may note, the R region amplified in brain tissues from patients 3A, 5A, 6A and 7A yielded sequences identical to the *Mad-4* strain. On the other hand, three brain samples from patients 8A, 9A and 10A showed multiple rearrangements, in addition to the deletion of the second TATA box, and displayed an A > G transition in nucleotide positions 108 and 208 (8A and 10A strains) and in nucleotide positions 106, 169 and 228 (9A strain).

The R-region sequences for patients 8A, 9A and 10A showed one 23-bp insertion in both tandem repeats, which is present in the archetype, but not in Mad-1. In patients 8A and 10A there were also two 15-bp insertions (5′-TAGTCCTTAATCACA-3′) similar to that found in M8AA (Martin *et al*, 1985).

Besides the previously mentioned 23-bp insertion, the 9A isolate showed a repeated 63-bp sequence which modifies the ‘B penta repeat’ region, characterised by the 5′-AGGGAAGGGA-3′ sequence. The characteristic 63-bp fragment containing an enhancer sequence, a sequence similar to

Table 2 Tissue samples positive (+) or negative (−) for the presence of JCV LT DNA in AIDS patients with (Group A) and without PML (Group B) and in HIV-negative patients (Group C)

Patient	*Brain*[a]	*Brain*[b]	*Lung*	*Kidney*	*Spleen*	*Liver*	*Lymph nodes*
Group A							
1A	+	+	−	+	+	−	na
2A	+	+	−	−	+	−	na
3A	+	−	+	−	−	−	na
4A	+	+	+	+	+	−	na
5A	+	+	+	+	−	−	−
6A	+	+	+	+	+	−	−
7A	+	+	+	−	−	−	na
8A	+[c]	−	−	−	−	−	−
9A	+	−	+	−	−	−	na
10A	+[c]	−	−	−	−	−	na
Total	10	6	6	4	4	0	0
Group B							
1B		−	−	−	−	−	−
2B		−	−	−	−	−	na
3B		+	+	+	−	−	−
4B		−	−	−	−	−	na
5B		+	−	+	−	−	−
Total		2	1	2	0	0	0
Group C							
1C		+	−	−	−	−	na
2C		−	−	−	−	−	na
3C		−	−	−	−	−	na
4C		+	−	−	−	−	na
5C		−	−	−	−	−	−
Total		2	0	0	0	0	0

[a]with active demyelinating lesions; [b]without demyelinating lesions; [c]JCV type 2; na: not available.

Table 3 Comparison of PCR results for the LT, R and VP1 regions of the JCV genome in the brain, lung, kidney and spleen tissue samples from AIDS patients with (Group A) and without PML (Group B) and from HIV-negative patients (Group C)

	Genomic region	*Brain*[a]	*Brain*[b]	*Lung*	*Kidney*	*Spleen*
Group A	LT	10	6	6	4	4
	R	7	3	4	1	2
	VP1	7	6	6	4	2
Group B	LT		2	1	2	0
	R		2	1	1	0
	VP1		0	0	1	0
Group C	LT		2	0	0	0
	R		0	0	0	0
	VP1		0	0	0	0

[a]with active demyelinating lesions; [b]without demyelinating lesions.

the V(D)J joining signal and to the transcription factor NF-1 site, was repeated three times. Therefore, this viral strain contained three copies of the immunoglobulin heptamer signal sequence, three copies of the NF-1 site and three copies of the enhancer core.

JCV genotype distribution
As concerns Group A samples, when tested with the inner-PCR specific for VP1, viral DNA was detected in seven brain sections from demyelinated areas and in six lesion-free sections, as well as in six lung, four kidney, and two spleen sections. Only one kidney section from a Group B patient was positive for JCV DNA within the VP1 region. None of the liver or lymph node sections analysed in this study proved to be positive for JCV DNA within the VP1 region. Thus, compared to the PCR for the LT region, the VP1-specific PCR proved to be less sensitive. Moreover, the distribution of the positive results for these two regions was not concordant with those obtained for the R region.

DNA sequencing of the VP1-positive samples revealed a close relationship of the isolates with the Mad-1 coding region. Three PML-affected brain sections from Group A patients (8A, 9A and 10A) contained viral sequences differing from the other isolates showing some point mutations already

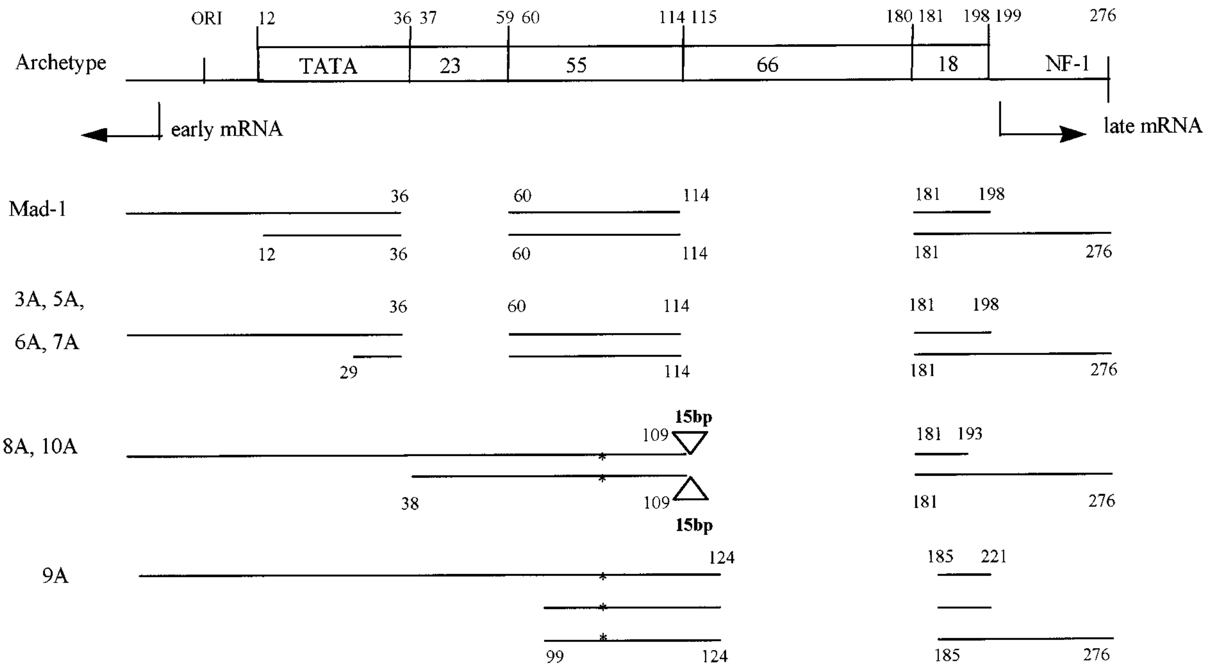

Figure 1 Schematic organisation of the regulatory regions of JCV isolates from brain tissues of PML patients, compared with archetypal JCV and Mad-1 strains. ORI indicates the origin of replication (at nucleotide 1 on genome map), TATA indicates TATA sequences and NF-1, the sites for NF-1 elements. Boxes lettered 23, 66 and 18 indicate the lengths of the sequences. Boxes for nucleotides 23 and 66 are not present in Mad-1. Triangles represent the insertion of sequences, the sizes of which are indicated below in base pairs (bp), and an asterisk represents a point mutation (A>G). Solid lines below sequences indicate repeat sequences. (Nucleotide numbering is based on Frisque *et al*, 1984).

Table 4 Nucleotide variations in the VP1 gene among the JCV 8A, 9A and 10A strains, amplified from PML brain sections, in comparison with type-1 and type-2 prototypal consensus sequences

JCV type	*Strain*	*2224*	*2227*	*2245*	*2260*	*2266*	*2269*	*2293*	*2311*	*2320*	*2326*	*2356*	*2386*	*2428*
Type 1	Mad-1	A	C	C	A	G	T	C	G	G	G	C	T	A
	H1	A	C	C	**T**	G	**A**	C	T	G	G	C	T	A
	N1	A	C	C	**T**	G	T	C	T	G	**A**	C	T	A
	9A	A	C	C	**T**	G	**A**	C	T	G	**A**	C	T	A
Type 2	GS/B	G	T	T	T	A	T	G	T	A	G	T	C	G
	8A, 10A	G	T	T	T	A	T	G	T	A	G	T	C	G

indicated in literature (Table 4) (Agostini *et al*, 1996a). It is interesting to observe that the VP1 DNA sequence for patient 9A was identifiable as JCV type 1, but with some unique point mutations. In fact, this isolate contained a A > T substitution in position 2260, observable in both the H1 and N1 strains, a T > A substitution in position 2269, which is observable in H1, but not in the N1 strain, and a G > A substitution in position 2326, as found in N1, but not in the H1 strain.

As reported in Table 4, the VP1 region DNA amplified from specimens from subjects 8A and 10A had the same mismatches observed in the GS/B strain and were therefore identifiable as JCV type 2. It is interesting to note that in these two cases, JCV LT DNA was detected only in the brain tissue, but not in any of the extraneural samples.

Discussion

Several recent studies have attempted to define the distribution of JCV in brain and extraneural tissues of patients with and without PML, often obtaining controversial results (Chester *et al*, 1983; Loeber and Dorries, 1988; Arthur *et al*, 1989; Elsner and Dorries, 1992; White *et al*, 1992; Ault and Stoner, 1994; Newman and Frisque, 1997). In this report, we have presented the results of a study aimed at assessing the presence and the characteristics of JCV in brain and in extraneural tissue samples collected at autopsy from AIDS patients with and without PML and from HIV-negative subjects. Among the group of samples from AIDs patients with PML, JCV conserved LT region DNA was detected in all the brain sections showing the characteristic signs of demyelination, and less frequently, in lesion-free brain tissue specimens. These results are in keeping with previous reports by Ueki *et al* (1994) and Vago *et al* (1996), who have demonstrated by *in situ* PCR and *in situ* hybridisation respectively, the presence of JCV DNA in oligodendrocytes at some distance from lesions. The results also confirm a diffuse presence of JCV in the brains of PML patients (Loeber and Dorries, 1988; Moret *et al*, 1993; Ferrante *et al*, 1995; Vago *et al*, 1996).

The presence of JCV in various tissues, including the liver, kidney, lymph nodes, lung and spleen has been already observed by others in a limited number of adult AIDS patients with PML (Grinnel *et al*, 1983; Houff *et al*, 1988; Monaco *et al.*) and in HIV-negative children affected by PML (Grinnel *et al*, 1983; Newman and Frisque, 1997). In our experiments, JCV DNA was also found in the lung, kidney and spleen tissues from PML patients, but not in liver and lymph node tissue sections. The negative results we obtained in the case of lymph node tissue specimens could be due to the very limited number of samples available, while it should be noted that reports of JCV in liver samples refer to HIV-negative subjects affected by PML (Grinnel *et al*, 1983; Newman and Frisque, 1997).

As concerns the samples from the non-PML subjects (Groups B and C), JCV DNA was found in brain sections collected from two AIDS patients and two HIV-negative subjects and also in the kidney sample from two subjects with AIDS and in the lung tissue from one AIDS patient. No JCV LT DNA was detected in the tissue samples from extraneural organs in the case of the HIV-negative subjects.

On the whole, in this study, the distribution of JCV in extraneural tissues collected from AIDS patients affected by PML showed a relatively high prevalence of JCV DNA in lung tissues. These data indicate that JCV can reach the respiratory tract, thereby lending support to the hypothesis that JCV infection can be acquired by an oral route (Shah, 1996).

Besides the prevalence of JCV infection, we evaluated the distribution of JCV variants in the samples collected from the different patient groups, using nucleotide sequence analysis. Given that the simultaneous presence of different viral strains in the same samples has been demonstrated by other researchers, we employed a long PCR for amplification of the R and VP1 regions, in order to increase the probability of analysing different regions of the same viral strain (Martin *et al*, 1984; Agostini *et al*, 1996a). However, probably due to the hypervariability of some JCV genomic regions, we observed lower sensitivity in the detection of the R and VP1 regions using this approach, as have other researchers (Telenti *et al*, 1990; Gibson *et al*, 1993; Stoner and Ryschkewitsch, 1995).

All the R region sequences amplified from the various tissues collected from the AIDS patients with and without PML, showed a rearranged feature. The detection of rearranged R region sequences also found in two kidney samples, conflicts with the hypothesis that the virus found in urine and in the kidney shows an archetypal R region and that the rearranged forms may be derived from the archetypal virus as a consequence of still largely unknown events (Loeber and Dorries, 1988; Yogo *et al*, 1990; Major and Ault, 1995; Ault, 1997). However, there have also been recent reports of the presence of PML-rearranged sequences in urine and in the kidney (White *et al*, 1992; Kitamura *et al*, 1994; Mediati *et al*, 1997).

The majority of the R-region amplified products showed rearranged sequences identical to the JCV Mad-4 strain, while three isolates obtained from demyelinated areas of PML brains, showed a different organisation. In two of these isolates, there was a duplicate 15-bp insertion, as already observed in other strains (Martin *et al*, 1985). The other sample showed an unusual R region with a 63-bp sequence containing a NF-1 binding site sequence, which was repeated three times. Although the

significance of this variation is unclear, it is known that NF-1 is a transcription factor that stimulates viral replication and thus a repetitive NF-1 sequence could indicate a signal amplification affecting the replication of this particular strain. This strain also lacked the two 'B penta repeat' motifs. This motif is known to function as an activator of the early JCV promoter and as a represssor of the JCV late promoter (Raj and Khalili, 1995). It has been demonstrated that mutations within the penta region affect DNA replication (Chang *et al*, 1994).

Various JCV genotypes have been identified in recent years on the basis of the nucleotide sequences of coding regions (Ault and Stoner, 1992; Iida *et al*, 1993; Agostini *et al*, 1996a). Using a similar approach in this study, we found a higher prevalence of type 1 JCV with Mad-1 strain-like features in the brain tissues and in the extraneural tissues collected, but particular nucleotide variations were observed in the VP1 DNA amplified from the brain sections of patients 8A, 9A and 10A. The isolate obtained from patient 9A, was characterised as type 1 JCV, but with some base mutations that characterise it as a variant with intermediate features between the H1 and N1 strains (Martin *et al*, 1985). The nucleotide sequence of the VP1 region amplified from the brain tissues of patients 8A and 10A was homologous to that of the GS/B strain, a JCV type 2, which was first isolated in Europe (Loeber and Dorries, 1988). Extraneural tissues from these two Group A patients were negative for JCV DNA and therefore we cannot establish whether a dual infection with both types 1 and 2 occurred in these patients.

The data presented here indicate that JCV is widely diffused in the brain and in many extraneural organs of AIDS patients with PML and to a lesser degree in AIDS patients without PML. As the patients in these two groups did not differ in terms of the level of immunosuppression at the time of death, one may justly suspect that other factors besides immunosuppression are contributing factors at work in determining the more widespread infection with JCV observed in PML patients.

The nucleotide variations involving both genome regions studied and observed only in PML-affected brain tissues, permit us to presume that variations involving different regions of the viral genome may actually represent additional risk factors for the development of PML in immunosuppressed individuals.

Materials and methods

Patients and samples

Tissues obtained at autopsy from ten AIDS patients with PML (Group A), five AIDS patients without PML (Group B) and five patients who died of diseases unrelated to AIDS (Group C) were formalin-fixed and paraffin-embedded.

The mean age of the AIDS patients was 37 years (range: 28–69 years) and that of the HIV-negative subjects was 74 years (range: 67–82 years). The mean $CD4^+$ count was 61 cells for Group A (range: 9–170) and 37.4 cells for Group B (range: 1–132). It was not evaluated for the Group C. Among Group C subjects, two had died of cerebral stroke with brain infarction, and the other three, of acute ischaemic heart attack.

Two 5-μm sections from the brain, lung, spleen, kidney and liver of each subject were analysed, whereas lymph node samples were collected only from three patients in Group A, three in Group B and one in Group C. For each Group A patient, two CNS blocks were selected, one from a demyelinating area in the parietal lobe or cerebellum and one from an area without demyelinating lesions in the frontal lobe of the brain. In the case of Groups B and C, samples were obtained from the frontal lobe.

All of these sections were cut using disposable blades and to avoid cross-contamination, the tissue sections from Groups B and C were cut using a microtome located in a different room than that used for Group A.

The histological examination of all tissue sections was routinely performed using hematoxylin-eosin staining, and luxol-fast-blue staining was employed for the evaluation of myelin damage in brain tissue sections.

DNA preparation and long n-PCR

DNA was obtained as previously described (Ferrante *et al*, 1995). Briefly, two 5-μm sections were deparaffined and incubated in digestion buffer at 56°C with proteinase K and, after phenol-chloroform extraction, the DNA was resuspended in distilled water and 5 μl of this solution were used in the PCR mix.

A nested polymerase chain reaction (n-PCR) was used. An outer L-PCR was performed using a set of primers amplifying a fragment of about 5 Kb. The procedure was carried out in a total volume of 50 μl containing 800 μM of each dNTP, 1.5 mM $MgCl_2$, 2.6 U Expand High Fidelity PCR system® (Boehringer Mannheim GmbH, Mannheim, Germany) with the appropriate buffer (500 mM Tris-HCl, pH 9.2, 160 mM $(NH4)_2SO_4$, 15 mM $MgCl_2$), and 50 pmol of the primers JRE1 (nt 4989-5009) and JC2 (nt 4573-4592). The enzyme was composed of a mix containing *Taq* DNA and *Pwo* DNA polymerases. *Pwo* DNA possessed $3'-5'$ exonuclease proofreading activity that results in a fidelity of DNA synthesis increased threefold, compared to *Taq* DNA polymerase.

After initial denaturation at 94°C for 2 min, a step consisting of ten cycles at 92°C for 20 s, 58°C for 30 s and 68°C for 1 min, was followed by a step with extension prolonged by 20 s with each cycle, for 20 cycles. Five microliters of the outer-PCR product were added to the inner PCR reaction mixture. This reaction mixture contained 200 μM of each dNTP,

10× reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM.KCl, 15 mM $MgCl_2$), 1 mM $MgCl_2$, 2 U of *Taq* polymerase Takara (Takara Biomedicals Shuzo Co, Ltd, Shiga, Japan) and 20 pmol of each specific set of primers (see Table 1).

The LT region (nt 4255-4427) was amplified using PEP-1 and PEP-2 primers, which yielded a fragment of 173 bp. The VP1 region (nt 2107-2500) was amplified using VPN3 and VPN4, yielding a fragment of 394 bp and the primers JR1 and JRN were used to amplify a fragment of about 313 bp (nt 5087-301) flanking the R region (nucleotide numbering is based on prototype Mad-1 from Frisque *et al*, 1984).

Strict precautionary measures were taken to avoid contamination and all PCR reactions were performed using one negative and one positive control.

The PCR products were detected by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Direct DNA sequencing
In order to perform direct sequencing of the R- and VP1- PCR products, modified 5′-biotin-labelled inner forward primers and 5′Cy-fluorescin-labelled reverse primers (Pharmacia Biotech, Uppsala, Sweden) were used for the amplification and sequencing reactions, respectively.

After the immobilisation of the biotinylated amplified products, the sequencing reactions were performed using the AutoLoad Solid Phase Sequencing kit (Pharmacia) according to the instructions of the manufacturer. The terminated sequencing products were run on 6% polyacrylamide gel containing 7 M urea (Ready Mix Gel, Pharmacia) onto an ALFexpress DNA sequencer.

Sequence analysis
Nucleotide sequences were aligned using the DNASIS V2.1 program (Hitachi Software Engineering America Ltd, San Bruno, California, USA).

Sequence changes were identified by comparing the data with corresponding typical JCV type 1 sequences such as the Mad-1 strain [J02227], Her-1(H1) [D11365] and N1 [D11358] and with European type 2 strain GS/B [M20322] (GenBank/EMBL Data library accession numbers are indicated in brackets).

Nucleotide sequence accession numbers
The sequences reported in this paper have been deposited in the GenBank database (accession numbers: AF047456 for the 8A strain; AF0443242 for the 9A strain; AF048698 for the 10A strain).

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