Co-infection with two JC virus genotypes in brain, cerebrospinal fluid or urinary tract detected by direct cycle sequencing of PCR products

Hansjürgen T Agostini¹, Caroline F Ryschkewitsch¹, Elyse J Singer² and Gerald L Stoner¹

¹Laboratory of Experimental Neuropathology, NINDS National Institutes of Health, Bethesda, Maryland 20892;
²Neurology Service, VAMC West Los Angeles, Los Angeles, California 90073, USA

The human polyomavirus JC (JCV), which exists in different geographically based genotypes, causes the central demyelinating disease known as progressive multifocal leukoencephalopathy (PML). A coding region recombinant JCV Type 1/Type 3 (Type 4) is excreted in the urine of some 16% of individuals in the USA. In addition, occasional 'crossovers' in viral DNA sequence at type-specific sites in the coding region occur between JCV genotypes amplified from PML brain. For recombination to occur requires the existence of two different genotypes in the same host. Here we provide evidence from direct cycle sequencing of PCR products that different genotypes of JCV can be found in a single tissue sample. After non-type-specific PCR amplification, cycle sequencing produced 'split bands' at type determining sites which were resolved into type or subtype-specific sequences by subcloning of the PCR products. PCR products with split bands at typing sites were found in two brain samples and in one cerebrospinal fluid (CSF) from AIDS patients with PML and in the urine of four immunocompetent individuals. This indicates that co-infection with two viral types does not depend on severe immunocompromise. Combinations of genotypes found were Types 1A & 1B, 1A & 2, 1B & 2 and 1A & 3. In one doubly infected patient the major JCV type excreted in the urine changed within 1 week.

Keywords: AIDS; polyomavirus; progressive multifocal leukoencephalopathy; recombination; urine; viral evolution

Introduction

JC virus (JCV) is a human polyomavirus which infects the majority of the human population worldwide during late childhood (Walker and Frisque, 1986). Its route of infection and mode of transmission are still unknown. However, following primary infection the virus persists in the kidneys and viral DNA can be detected by PCR in the urine of 30–50% of immunocompetent individuals above 30 years of age (Kitamura et al, 1990; Agostini et al, 1996). Replication of JCV in oligodendrocytes and astrocytes in the brain is strongly associated with immunosuppression and causes the fatal CNS demyelinating disease progressive multifocal leukoencephalopathy (PML) (for reviews see (Major et al, 1992) and (Berger and Concha, 1995)). PML affects about 5% of AIDS patients in Europe and the USA (Kuchelmeister et al, 1993) and is the AIDS defining illness in 25% of these cases (Berger and Levy, 1993). PML is usually fatal within 3 to 9 months, although up to 10% of patients may survive for a year or more without specific treatment.

The genes coding for the viral structural and regulatory proteins are transcribed divergently from the non-coding regulatory region. The synthesis of mRNA for the structural proteins VP1-3 and the agnoprotein is part of late viral transcription and is regulated by the early protein, T antigen (Frisque and White, 1992; Raj and Khaili, 1995). The regulatory region of JCV excreted in urine shows predominantly an unarranged (archetypal) configuration (Yogo et al, 1990) which likely represents the transmissible form of JCV. In contrast, regulatory regions from PML brains show individual rearrangements that are derived from this archetypal structure (Ault and Stoner, 1993). No association between these regulatory region rearrangements and the viral coding region genotype has been found (Ault and Stoner, 1993).
Viral coding region genotypes were defined by restriction fragment length polymorphism (RFLP) (Yogo et al., 1991) and comparative sequence analysis (Ault and Stoner, 1992; Iida et al., 1993) from Asian, European and American strains. In Europe JCV Type 1 predominates, although JCV Type 2 was found in a PML patient in Germany (Loebel and Dorries, 1988). In Asia JCV Type 2 is the only type which has been characterized. The African JCV strains constituting JCV Type 3 were recently described in the urine of Tanzanian HIV-1 positive patients (Agostini et al., 1995). Unlike Type 1 and Type 2, in which the sequence of the non-rearranged archetypal regulatory region is not associated with a particular genotype, the regulatory region of Type 3 strains can be clearly distinguished by the presence of deoxycytidine at position 133 (Agostini et al., 1995).

In our experience a 129-bp fragment from the middle portion of VP1 amplified by PCR using the primers JLP-1 and JLP-4 has proved to be very useful for routine diagnosis of JCV infection in clinical samples (Agostini et al., 1995; 1996; Stoner et al., 1996b). It is possible to differentiate all major JCV types within this short region of the major capsid protein. JCV Type 1 strains can be divided into subtypes based on additional sequence differences (Agostini et al., 1996). This portion of VP1 is also a site of rare coding region deletions (Stoner and Ryschkewitsch, 1995).

About two thirds of strains circulating in the USA are JCV Type 1A or 1B as determined from virus excreted in urine (Agostini et al., 1996). The prototype strain, JCV(Mad-1), is a Type 1A, but overall JCV Type 1B strains are about twice as frequent as JCV Type 1A. JCV Type 2 was detected in 18% of JCV-positive urines in the USA. The recombinant between Type 1 and Type 3 (termed Type 4) was shown to be nearly as prevalent in the USA as JCV Type 2 (Agostini et al., 1996). JCV Type 4 is especially interesting from an evolutionary point of view (see Discussion). The majority of its sequence is similar to the Type 1 consensus sequence with the exception of 142 bp within the VP1 gene which includes four Type 3-like sites ([Agostini et al., 1996] and unpublished data). In addition to the highly prevalent JCV Type 4, the most striking evidence for coding region recombination is the existence of a unique strain (#201) in which a Type 1-like fragment of 136 bp replaces a Type 2 sequence within the VP1 gene (Ault and Stoner, 1992).

Here we provide evidence that double infections with two different types or subtypes of JCV can occur in the urinary tract of control individuals or MS patients and in the CNS of PML patients. Direct cycle sequencing of PCR products from the VP1 coding region results in characteristic 'split bands' at typing sites in these individuals. Subcloning of the PCR products provides confirmation that these tissues are infected with two separate viral types or subtypes.

**Results**

**Co-infection with two JCV types in PML CSF**

The JLP-1 & 4 fragment (1769–1897) includes a total of six typing sites for JCV Types 1–4 as shown in Figure 1. Subtypes 1A and 1B of JCV can be distinguished at positions 1843 and 1850. Direct cycle sequencing of the 129-bp PCR product usually allows unambiguous determination of type. None of these typing positions was prone to cycle sequencing artifacts.

Individual samples were proposed to be simultaneously infected by two JCV types when split bands were found at type-specific positions. In general, corresponding type-specific bands were of similar intensity but less intense than non type-specific bands from the same reaction. In order to confirm that these bands were not artifacts but originate from the previously defined JCV types, PCR products were subcloned and again directly sequenced after screening clones by PCR with primers JLP-1 & 4.

An example of split bands as a result of cycle sequencing is shown in Figure 2, middle lane. From a total of 13 CSF samples positive for JCV with primers JLP-1 & 4, a sequence with split bands was observed only in the CSF of a 47-year-old male AIDS patient. Ligation of the mixed product produced clones carrying plasmids with either Type 1A or Type 1B inserts (Figure 2, outer lanes). In addition to the typing sites at 1843 and 1850, a unique mutation from deoxycytidine to deoxyadenosine

<table>
<thead>
<tr>
<th>Type 1A</th>
<th>TAAAACTGAGGTTATAGGCGCTAACAAGTTGATGAAATGACA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>G</td>
</tr>
<tr>
<td>Type 2</td>
<td>T</td>
</tr>
<tr>
<td>Type 3</td>
<td>C</td>
</tr>
</tbody>
</table>

Figure 1 Partial VP1 gene as amplified by primers JLP-1 and JLP-4 (Table 1). The presence of four major typing sites (1804, 1815, 1869, 1870) allows differentiation of JCV Types 1, 2 and 3. JCV Type 1 can be further subdivided into Type 1A and Type 1B at positions 1843 and 1850. A variable nucleotide at position 1837 is a possible subtyping site for JCV Type 2 strains. Y=T or C, R=A or G. Numbering is based on JCV(Mad-1) (Frisque et al., 1984). Dashes indicate identity with the JCV Type 1A sequence.
was found at position 1804. This nucleotide change could be attributed to the JCV Type 1B strain after subcloning.

Co-infection with two JCV types in PML brain

From a total of 31 frozen brain biopsy or autopsy samples which tested positive for JCV by PCR, the phenomenon of split bands at typing sites was detected in two patients (6%). The sequences of the subcloned PCR products in a woman with Hodgkin’s disease were consistent with JCV Type 1A and Type 1B in the autopsy material (Figure 3).

In the case of a 36-year-old male AIDS patient the DNA sequence of PCR reaction products from the VP1 and T antigen coding regions was consistent with the presence of both JCV Type 1A and Type 2 (Figure 4). Beside the major typing sites at positions 1818 and 1870 and the subtyping sites at positions 1843 and 1850, the sequence analysis of the PCR product showed a split band at 1837. Subcloning demonstrated that ‘C’ at 1837 was associated with the Type 2 sequence. Deoxycytidine at this position may define the variant of JCV Type 2 strains found in Europe and the USA (Iida et al., 1993). The type distinction of JCV Type 1 and Type 2 in the T antigen gene is based on the comparison between the prototype strains JCV(Mad-1) and JCV(GS/B), respectively. Although the type-specific sites of JCV are not yet definitely established for this region, split bands were detected at positions 3020 [T in JCV(Mad-1); A in JCV(GS/B)], 3035 [C in JCV(Mad-1); T in JCV(GS/B)] and 3053 [C in JCV(Mad-1); T in JCV(GS/B)] (Figure 4, right lane). Thus, the phenomenon of split bands at typing sites determines that JCV type is not limited to products amplified from VP1, but reflects the existence of type-specific or strain-specific nucleotide changes anywhere in the genome.

Examples of double infection in urine

Studies in our laboratory investigating urinary excretion of JCV in different cohorts included a total of 75 positive individuals (Agostini et al., 1996 and unpublished data) of which four (5%) were infected with more than one JCV type based on the appearance of split bands at typing sites after cycle sequencing.

In the case of a 40-year-old MS patient two urine samples were examined that were taken one week apart. Both contained JCV DNA but the type amplified by PCR changed from JCV Type 3 in the first urine sample to predominantly JCV Type 2 in the second sample (Figure 5). Split bands at Type 2 and Type 3 defining sites were found in the second specimen repeatedly after PCR and cycle sequencing with primers JLP-1 & 4. In the second specimen JCV Type 3-specific bands were much less intense on the autoradiogram than those for Type 2. However, this Type 2 sequence was not detected in the first specimen from this patient. A shift of type of the predominant virus in the urine appears to have occurred within one week.

Other individuals with two JCV types included a healthy 23-year-old man excreting viral DNA of JCV Type 1A and Type 2 (Figure 6) and a 50 year old
male MS patient with Type 1B and Type 2. JCV subtypes 1A and 1B could be distinguished in a urine specimen from a 38-year-old male outpatient from a general medical clinic (Figure 7). Subcloning of the PCR products generated two sets of clones with either one or the other type-specific JLP-1 & 4 fragment, as demonstrated by reamplification and cycle sequencing of the inserts (Figures 6 and 7, outer lanes). Split bands were separated in the subcloned and reamplified sequences. Attempts to amplify two genotypes by complete genome PCR in doubly infected samples did not yield virus-specific products.

**Discussion**

JCV genotypes have been defined in the V-T intergenic region (Ault and Stoner, 1992) and in the VP1 gene (Iida et al, 1993). More recently, a short fragment amplified from VP1 has consistently differentiated all four major JCV genotypes and the two subtypes of Type 1 (Agostini et al, 1996). While regulatory region rearrangements are important for generating neurotropic viral variants, we believe genotypes based on coding region variations may also prove to influence viral targeting, expression and/or pathogenicity. There is no consistent correlation between regulatory region rearrangements or nucleotide variations in the archetypal regulatory region right of origin and the Type 1 and Type 2 genotypes (Ault and Stoner, 1993; Agostini et al, 1996). Therefore, it is preferable to examine the VP1 coding region in order to identify double infection with two different genotypes. Previously, two differently rearranged regulatory regions have been observed in the same PML brain, but these are likely to have been variations on one infecting coding region genotype (Martin and Foster, 1984; Yogo et al, 1994).
During direct cycle sequencing of PCR products from CSF, brain tissue and urine, we observed the occurrence of 'split bands'. These split bands were easily distinguishable from cycle sequencing artifacts in which bands of similar intensity cross all four nucleotide lanes. Here the ambiguous reading was confined to two nucleotides at type determining sites. Subcloning of the mixed products was able to separate type-specific sequences. The 129-bp fragment within the VP1 gene amplified by primers JLP-1 & 4 provided a convenient target within which to observe split bands after direct cycle sequencing. Based on the knowledge of type-determining sequence variations within the coding region of JCV strains, direct cycle sequencing of PCR products can be considered a suitable method to screen for co-infection with multiple JCV strains in a tissue or body fluid. The standard approach to detection of double infections would be to analyze large numbers of sequences cloned directly from infected tissues of many different individuals. This approach would be prohibitively time consuming.
Type 4 strains have now spread widely in the population of the USA (Agostini et al., 1996). This together with the presence of three additional single site differences between the Type 1 and Type 4 sequences within the V-T intergenic region (Figure 8a, positions 2227, 2356 and 2661) indicate that the initial recombination event did not take place recently. The wide distribution of Type 4 strains in the USA strongly suggests that the recombination event was associated with a distinct biological advantage. The Type 1/Type 3 recombination introduced a change in the relative net-charge of VP1 (Figure 8b). The implications of double infections for recombination between types and thus for the ongoing evolution of this DNA virus are evident.

The frequency of double infections was about 5% in urine and CNS tissue of PML patients. The fact that different types of the virus can be detected in the urine at different times as shown for an MS patient carrying JCV Type 2 and Type 3 suggests the possibility that the infecting virus types may be latent in different sites within the urinary tract and that the level of viral reactivation and excretion is to some extent independently regulated. The majority of the specimens included in these studies were single samples from each individual. The most common pairing of strains infecting the same tissue are JCV Types 1A & 1B and Types 1 & 2. This correlates generally with the frequency of these types in a control group from a general medical clinic (Agostini et al., 1996). However, it should be noted that JCV Type 4, although found nearly as frequently as JCV Type 2, has not yet been observed in doubly infected tissue samples. More data on double infections will be necessary to determine whether this absence reflects an actual biological behaviour.

The importance of viral genotypes for understanding biological behaviour is supported by several lines of evidence. First, different genotypes predominate in different Old World populations and thus they reflect viral evolution and epidemiology. Second, the frequency of JCV Type 2 strains is significantly higher in PML tissue than in JCV-positive urines of non-PML individuals, indicating a difference in the biological behaviour of JCV genotypes (Stoner et al., 1996a). Finally, as discussed above, the ability of a Type 1/Type 3 recombinant in VP1 (Type 4) to spread throughout the population indicates a definite biological advantage on the part of the recombinant. An analysis of the complete JCV Type 4 genome is in progress.

Co-infection with different genotypes of JCV does not appear to be correlated with a specific disease. Since it can be found in the CNS of AIDS patients and Hodgkin’s disease patients, and also in urine of MS patients and healthy individuals, the presence of two different JCV genotypes seems to be
independent of the immune status of the host. With regard to the reactivation of polyomaviruses in the kidney, previous studies with AIDS patients have shown that the excretion rate of the other human polyomavirus BKV, but not JCV, increases with increasing immunodeficiency (Markowitz et al., 1993; Sundsfjord et al., 1994).

How do double infections originate? It is possible that the inoculum these individuals received was itself doubly infected. On the other hand, such double infections could arise (and may originally have arisen) from nearly simultaneous exposure to different infectious individuals carrying different JCV types or subtypes. If the second exposure occurred during the interval between acquisition of the first infection and the development of a protective immune response, then a double infection might be established. Alternatively, a second JCV infection might be acquired during periods of transient non-specific immunosuppression.

Patients and methods

Samples

Brain biopsy samples (15–20 mg) were lysed overnight at 55°C in 100 μl buffer containing 0.2 mg/ml proteinase K (BRL, Gaithersburg, MD) and 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM MgCl₂, 10% (v/v) gelatin, 0.45% (v/v) NP40 and 0.45% (v/v) Tween 20. DNA from CSF was extracted by adding NP40 at a final concentration of 0.05% (v/v) and incubating at 56°C for 30 min. Brain and CSF samples were obtained as previously described (Stoner and Ryschkewitsch, 1995). 25–50 ml of fresh urine was centrifuged, the pellet washed with PBS and lysed with proteinase K in lysis buffer in a total volume of 100–200 μl depending on the size of the pellet. After proteinase K digestion the samples were boiled for 10 min and stored at −20°C. Urine samples were obtained from unselected adult donors in a general medical clinic and from healthy volunteers (Agostini et al., 1996b) or from patients with multiple sclerosis (Stoner et al., 1996b).

PCR

Primers JLP-1 & 4 and JTP-1 & 2 were used for PCR amplification of a 129-bp sequence within the VP1 coding region and a 141-bp sequence within the T antigen coding region, respectively. For details on the oligonucleotides see Table 1. Lysed samples (5 μl of urine and brain, 10 μl of CSF) were added to the reagent mixture containing 1.5 mM Mg²⁺ (10 × PCR Buffer, Perkin Elmer Cetus, Norwalk, CT), 300 nM of each primer, dNTPs (200 μM) and 4.5 U of UITma DNA Polymerase with 3'-5' proofreading activity (Perkin Elmer Cetus). Hot start was performed by adding dNTPs or the DNA polymerase to a total of 100 μl after initial heating at 94°C for 3 min. An alternative hot start method used wax beads (Invitrogen, San Diego, CA) from which magnesium is released at high temperature into the magnesium-free reaction buffer. The 50-cycle program (Thermal cycler 480, Perkin Elmer Cetus or PTC-100, MJ Research, Watertown, MA) included annealing for 1 min at 55°C (three-step) or 63°C (two-step) for JLP-1 & 4 and 55°C for JTP-1 & 2, extension for 1 min at 72°C (three-step) and denaturation for 1 min at 94°C. After a final 10 min extension at 72°C the reaction was stopped at 4°C. PCR products were cleaned for cycle sequencing and subcloning by preparative agarose gel electrophoresis followed by the Qiagen gel extraction procedure (Qiagen, Chatsworth, CA). Methods used to amplify the complete genome by PCR were described in detail previously (Agostini and Stoner, 1995).

Subcloning of PCR products

PCR products with distinguishable double bands at typing positions within the JLP-1 & 4 amplified fragment (see Results) were ligated into the pN0TA/T7 plasmid using the Prime PCR Cloner (5 Prime 3 Prime, Boulder, CO). The plasmid vector was introduced into competent E. coli using heat shock at 42°C. Clones with ampicillin resistance and missing x-complementation on LBagar plates coated with isopropyl-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were transferred to a second plate for overnight culture. To detect clones carrying plasmids with the partial VP1 gene insert, bacterial cultures were partially scraped from the plate, resuspended in 30 μl of sterile water and heated for 15 min at 100°C. 5 μl of the bacterial lysate was used for PCR with primers JLP-1 & 4 as outlined above.

Cycle sequencing

Primers (100 ng) were end labeled with 60 μCi of 33P-ATP (Amershams, Arlington Heights, IL) in a total volume of 25 μl. Radiolabeled primer (1.8 ng) was combined with 2–5% of the cleaned template, DNA polymerase and the chain-terminating dideoxynucleotides (SequTherm cycle sequencing, Epicentre Technologies, Madison, WI). The initial denaturation for 1 min at 95°C was followed by 30 cycles of 30 s at 95°C for denaturation and 1 min at

Table 1 JCV specific primers

<table>
<thead>
<tr>
<th>Code</th>
<th>5'-3'</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>JLP-1</td>
<td>CTGATGCTGAGGCTTGC</td>
<td>1769-1790</td>
</tr>
<tr>
<td>JLP-4</td>
<td>ATGAGACTGTTGCCTGC</td>
<td>1897-1876</td>
</tr>
<tr>
<td>JTP-1</td>
<td>CAGCTTAGTTTTCAGG</td>
<td>2990-3011</td>
</tr>
<tr>
<td>JTP-2</td>
<td>CACCAAAACAAAGACAG</td>
<td>3130-3109</td>
</tr>
</tbody>
</table>

*numbering based on JCV(Mad-1) (Prieske et al., 1984)
65°C for annealing and elongation. Products were run on a 6% polyacrylamide gel containing 50% (w/v) urea (National Diagnostics, Atlanta, GA). After fixation in 12% (v/v) methanol+10% (v/v) acetic acid, the gels were transferred to 3 mm chromatography paper (Whatmann, Maidstone, England), dried under vacuum and exposed to X-ray film for 12–48 h.

**Reference sequences**

(GenBank/EMBL Data Library five-digit accession numbers are in brackets [ ]). Consensus sequences of the JLP-1 & 4 fragment for JCV Type 1 (including Type 1A and Type 1B) [U21842], JCV Type 2 [U21843] and JCV Type 3 [U21844] (Agostini et al., 1995); Complete genome of JCV(Mad-1) [J02227] (Frisque et al., 1984); JCV(GS/B) [M20322] (Loeber and Dörries, 1988). Numbering based on JCV(Mad-1) sequence.

**Acknowledgements**

This work was in part supported by the Deutsche Forschungsgemeinschaft (grant Ag 19/1-1). We thank Cindy Kauffman for collecting urines. We also thank Dr RW Baumhefner and Dr WW Tourtellotte for urines from MS patients. The support of Dr Henry deF Webster is gratefully acknowledged.

**References**


