Archetypal and rearranged sequences of human polyomavirus JC transcription control region in peripheral blood leukocytes and in cerebrospinal fluid

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Two forms of human polyomavirus JC (JCV) genome are known based upon the structure of the transcriptional control region (TCR) of the virus: the archetypal form, which is commonly detected in urine, and the rearranged form, which was first detected in brain tissue from progressive multifocal leukoencephalopathy (PML) patients. The latter actually includes a group of TCR variants that, relative to the former, are characterized by various deletions and/or duplications. The aim of this study was to establish whether or not a correlation exists among the TCR type, the spreading of the virus within the host and its ability to cause PML. JCV TCR sequences from peripheral blood leukocytes (PBL) and cerebrospinal fluid (CSF) obtained from various groups of patients were compared. JCV with archetypal TCR was detected in CSF and PBL specimens from patients without neurological disorders or who eventually received a diagnosis of a non-PML neurological disorder. Rearranged TCR sequences were detected in all the CSF and PBL specimens from PML patients. The high similarity observed between the TCR structure detected in PBL and CSF specimens from individual patients could strengthen the hypothesis that PBL has a role in spreading JCV to the brain. Moreover, heterogeneous TCR patterns have been shown in individual PBL specimens from PML patients. This supports the hypothesis that, in PBL, JCV may replicate and undergo rearrangements of the TCR. The detection of JCV DNA by PCR in CSF independently from PML, although rare, could suggest that this assay is not sufficient for a virological diagnosis of PML. Further studies are required to assess the usefulness of quantitative assays or TCR typing in combination with PCR for diagnostic purposes.

Introduction

Human polyomavirus JC (JCV) infection is widespread in the human population as inferred by serological surveys (Walker & Padgett, 1983). However, the route of transmission and the site of initial infection in the body have not yet been detected, mainly because of the asymptomatic nature of the primary infection.

More information, although this is still incomplete, is known about the events that follow initial exposure. In fact, it has been documented that JCV persists in the kidney, in the bone marrow and haematopoietic system, in peripheral blood leukocytes (PBL) and also in the brain (Chester et al., 1983; Houff et al., 1988; Elsner & Dörries, 1992; White et al., 1992; Dörries et al., 1994). Virus reactivation may ensue under different conditions such as an impairment of T cell-mediated immunity or the influence of cytokines on viral gene expression (Atwood et al., 1995; Chang et al., 1996). In the kidney, reactivation of the latent JCV may result in its asymptomatic shedding in the urine of both immunocompromised and immunocompetent individuals (Markowitz et al., 1993; Azzi et al., 1996). In the brain, JCV lytic infection of myelin-producing oligodendrocytes causes progressive multifocal leukoencephalopathy (PML), a fatal progressive demyelinating disease. This disease occurs in patients with severe and long-lasting
immunological impairment and is increasingly recognized in AIDS patients (Berger & Concha, 1995). Thus, JCV may cause both latent and productive infections in the urinary tract as well as in the brain and, in addition, it may persist in leukocytes. The JCV transcription control region (TCR) has a central role in virus multiplication. This non-coding regulatory region contains a bi-directional promoter/enhancer which directs early transcription of large and small T antigens from one strand and, after DNA replication, directs late transcription of the structural proteins from the other strand. The JCV TCR detected in the urinary tract is distinguishable from that of JCV detected in the brain, whose prototype is Mad-1 (Walker & Frisque, 1986). The first form, known as the archetype (Yogo et al., 1990), is thought to be the form of virus that circulates among the asymptomatic population. The archetype could be the source of the forms found in the brain, produced via deletions and sequence duplications, that give variants that are more active and with a new tissue tropism (Yogo et al., 1990; Flägstad et al., 1991; Ault & Stoner, 1993). This conclusion is supported by the observation that the JCV TCR nucleotide sequence from PML brains is highly variable, in contrast with those from urine and kidney which are nearly identical. Recently, however, the results of the analysis of JCV TCR detected in persistently infected brain and kidney tissues have suggested the possibility that a limited number of JCV TCR subtypes, with rearranged as well as archetypal structure, circulate in different geographical regions (Elsner & Dörries, 1998). In lymphocytes, the few available data suggest the presence of rearranged as well as archetypal forms (Tornatore et al., 1992; Dörries et al., 1994; Azzi et al., 1996).

In this study, we have compared JCV TCR sequences from cerebrospinal fluid (CSF) and PBL specimens from different individuals in order to better understand how JCV spreads within the host and the role of rearrangements in the JCV TCR. This knowledge may contribute to the detection of significant diagnostic and prognostic virus markers.

Methods

Patients and samples. The JCV DNA-positive specimens analysed in this study were obtained from 22 AIDS patients and seven immunodeficiency virus (HIV)-negative individuals. Seventeen of the AIDS patients (patients 1–17) had neurological disorders which could be consistent with PML at the time of virological assay and one of the HIV-negative patients (patient 18) had tuberculous meningitis. From this group of patients, we analysed 15 CSF specimens (CSF.1, CSF.3–CSF.12, PBL.17); only PBL specimens were available from patients 2, 13 and 15. CSF and PBL specimens were obtained 15 days to 1 month after the onset of neurological disorders. Eleven PBL specimens were obtained from five AIDS patients and six HIV-negative patients [three bone marrow transplant (BMT) patients and three blood donors] without neurological disorders.

Diagnosis of PML was suspected by clinical features, such as personality changes and any one or a combination of hemiparesis, visual field defects, cortical blindness, aphasia, ataxia and disartria, and by cranial magnetic resonance imaging showing widespread demyelinating lesions mainly of the cerebral hemispheres, but also of the brainstem and cerebellum in some patients, without mass effect and without enhancement. The CSF did not show pleocytosis and the protein content was normal or slightly increased.

In addition to JCV detection, in the CSF specimens, PCR assays for HIV, cytomegalovirus, herpes simplex virus-1, herpes simplex virus-2, varicella-zoster virus and Epstein–Barr virus were performed as well as the latex agglutination test for cryptococcal polysaccharide (at the Laboratory of Microbiology, Virology and Immunology of the Azienda Ospedaliera di Careggi, Firenze, Italy).

PCR amplification of viral DNA from CSF and PBL. Prior to the amplification reaction, the clinical samples were pretreated as previously described (Azzi et al., 1996). Briefly, DNA from PBL samples, after separation on a Ficoll–Hypaque gradient and subsequent cellular lysis, was extracted by the phenol–chloroform method; for CSF, samples were boiled for 10 min followed by ethanol precipitation. DNA (2 µg) extracted from PBL and 5 µl DNA extracted from CSF were added to the PCR mixtures. A nested PCR was performed for the amplification of the JCV TCR, as already described (Azzi et al., 1996). Briefly, the external primers BKTT1 and BKTT2 (Sundsfjord et al., 1990) were utilized for the amplification of a fragment about 700 bp long and JC1 and JC2 (Markowitz et al., 1991), the internal primers, amplified a fragment about 300 bp long within the previous one. PCR products were analysed by electrophoresis on an agarose gel containing ethidium bromide.

Restriction fragment length polymorphism (RFLP) analysis. The cloned TCR were amplified by PCR using the internal primers only (JC1 and JC2) and separately digested by the enzymes SstI and SpII as already described (Azzi et al., 1996). The first enzyme cuts the prototype of rearranged TCR, Mad-1, into three fragments of about 102, 98 and 117 bp, but the cleavage of other types of rearranged TCR may give rise to fragments of different sizes and the archetypal TCR is cleaved by the same enzyme into two fragments of about 125 and 175 bp. Moreover, Mad-1 is not cleaved by the enzyme SpII, whereas the archetypal TCR is cleaved into two fragments of about 176 and 124 bp by this enzyme.

Nucleotide sequence analysis. Sequencing of the TCR was performed directly after PCR amplification or after cloning of the PCR products. The nucleotide sequences were obtained by cycle sequencing on an ABI 373A automated DNA sequencer (Perkin-Elmer, Norwalk, Conn., USA). The nucleotide sequences were obtained by cycle sequencing (Amplicycle; Perkin Elmer), a modification of Sanger’s method (Sanger et al., 1977). Both strands were sequenced with primers JC1 and JC2, which were already used as inner amplification primers. Briefly, the samples, after PCR, were purified through a Qiaquick column (Qiagen). The cycle sequencing reaction was performed on 100 fmol purified DNA in a total volume of 8 µl containing: 6 µl cycling solution [50 mM Tris–HCl pH 8.9, 10 mM KCl, 2.5 mM MgCl₂, 0.25% (v/v) Tween 20, 20 µM primer JC1 or JC2, x-32P-labelled ATP (125–25 µCi) and 0.25 U AmpliTaq DNA polymerase] and 2 µl dNTPs mix [22.5 µM dGTP, 10 µM dATP, 10 µM dTTP and 100 µM dCTP] and one of the four terminators (80 µM dGTP, 600 µM dATP, 900 µM dTTP or 300 µM dCTP)]. Cycling protocol for the sequencing reaction was: 1 min at 95 °C, 1 min at 68 °C (JC2/JC1) and 1 min at 72 °C, for a total of 35 cycles. The reaction was stopped by the addition of 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.02% xylene cyanole FF). The resulting products were run on a polyacrylamide gel (6% polyacrylamide and 7 M urea). The gels were fixed, dried under vacuum and exposed to Kodak BioMax films for 3–6 days.

Molecular cloning. In order to investigate the existence of multiple variants in a single clinical sample, PCR products resulting from the
amplification of some PBL and CSF samples were cloned into pGEM-T vectors (Promega). Ten clones were examined for each clinical sample.

Computer analysis. Sequences were aligned with DNASIS software.

Results

We analysed JCV TCR sequences detected in a number of PBL and CSF specimens. As Table 1 shows, paired CSF and PBL specimens were obtained from seven patients (six AIDS patients and one HIV-negative patient with tuberculous meningitis), whereas CSF or PBL were only available from eight and three AIDS patients with neurological disorders, respectively. Determination of TCR patterns was performed only on PBL samples for patients without neurological disorders (five AIDS patients and six HIV-negative individuals). The TCR was amplified by PCR between nucleotides 5089 and 272 (relative to the genome of the prototype Mad-1) and sequenced. The obtained sequences were aligned with the archetypal sequence (Yogo et al., 1990) and divided in six regions, A–F, in order to highlight their similarity with the archetype or their rearrangements, as reported in the literature (Ault & Stoner, 1993).

Table 1 shows that the archetypal sequence was detected in JCV genomes from PBL of three blood donors, as immunocompetent individuals, of three BMT patients and of five AIDS patients without neurological symptoms. In one of these five AIDS patients, a second PBL specimen was obtained after 4 months. It was again JCV DNA-positive with an archetypal TCR. Moreover, an archetypal TCR was detected in three CSF samples (CSF.6, CSF.16 and CSF.18) from two AIDS patients with neurological disorders, which could be consistent with PML, and from an HIV-negative patient with tuberculous meningitis. The JCV TCR detected in PBL samples of the same patients (PBL.6, PBL.16 and PBL.18) was also archetypal.

Rearranged sequences were detectable in the JCV genomes from CSF and/or PBL of the other 15 AIDS patients with neurological disorders (Table 1).

To evaluate the possibility that JCV is carried to the brain by PBL, we have compared the TCR detected in paired specimens of JCV DNA from CSF and PBL of the same patient (CSF.1/PBL.1, CSF.12/PBL.12, CSF.14/PBL.14, CSF.17/PBL.17). In all cases examined, JCV from both specimens showed a TCR with a rearranged pattern. As shown in Fig. 1, in three cases, similar TCR rearrangements were detected in CSF and PBL of the same patient. In the remaining case (CSF.1/PBL.1), the TCR detected in CSF was like that of Mad-1. The TCR detected in PBL, however, was characterized by the complete deletion of regions B and D, as Mad-1, but showed only a partial duplication of region C and no duplication of regions A and E.

In order to evaluate whether different variants may be present in a single clinical sample, the JCV TCR sequences amplified from four CSF (CSF.6, CSF.11, CSF.16 and CSF.17) and three PBL specimens (PBL.1, PBL.2 and PBL.17), all from AIDS patients with neurological disorders, were cloned; 10 clones for each sample were analysed by RFLP and at least four of these were sequenced. The clones from the four CSF samples and from the PBL.1 sample gave similar results. All the clones from CSF.11 and CSF.17, as well as those from PBL.1, were of the rearranged type, whereas all the clones from CSF.6 and CSF.16 shared an archetypal structure. In contrast, the clones from the PBL.2 sample displayed a remarkable heterogeneity, from archetypal to Mad-1 structure, with intermediate rearrangements. Two patterns emerged from analysis of the clones from the PBL.17 sample, one like Mad-1 (two clones) and the

Table 1. Detection of rearranged or archetypal JCV TCR in CSF and PBL from various groups of patients

<table>
<thead>
<tr>
<th>Patients and clinical diagnosis</th>
<th>No. of patients (sample analysed)</th>
<th>Rearranged TCR</th>
<th>Archetypal TCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With neurological disorders</td>
<td>8 (CSF)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3 (PBL)*</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6 (CSF and PBL)</td>
<td>(4 + 4)†</td>
<td>(2 + 2)†</td>
</tr>
<tr>
<td>Without neurological disorders</td>
<td>5 (PBL)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>HIV-negative patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuberculous meningitis</td>
<td>1 (CSF and PBL)</td>
<td>0</td>
<td>(1 + 1)†</td>
</tr>
<tr>
<td>BMT recipients</td>
<td>3 (PBL)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Immunocompetent‡</td>
<td>3 (PBL)</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* CSF from these three patients was JCV DNA–positive but was not available for sequencing.
† Rearranged or archetypal sequences were detected in both CSF and PBL samples from the same patient.
‡ Blood donors.
second (eight clones) characterized only by the deletion of regions B and D; this latter pattern was also observed in a clone of the PBL.2 sample and it was similar to that of the W4 variant recently described (Elsner & Dörries, 1998) (Fig. 1.).

In Fig. 1, the diagrams of the rearranged TCR detected in this study are compared with the archetypal sequence. The TCR of the JCV genome in CSF.1, CSF.4 and CSF.17 samples was similar to that of the prototype strain Mad-1 as well as that detected in PBL.2 (clone 5) and PBL.17 (clone 30) samples. The other genomes, with rare exceptions, showed unique TCR sequences, having only a few features in common, such as the partial or complete deletion of region D and the constant presence of regions A and F. Moreover, C and E duplications were frequently observed. Most rearranged TCR sequences of JCV from CSF samples (10 out of 12) exhibited a pattern similar to the 'long duplicate' pattern described in the literature (Ault & Stoner, 1993) with duplications of regions C and E and deletion of region D. This pattern was also detected in five out of seven JCV strains from PBL.

Referring to a classification based on the differences within the TCR (Frisque & White, 1992), the TCR detected in CSF.1, CSF.4 and CSF.17, and in PBL.2 (clones 3 and 5), PBL.1 and
PBL.17 (clone 30) could be classified as TCR type I. All the others were of TCR type II.

In addition, few sporadic point mutations have been also detected, possibly caused by Taq polymerase errors. As already reported in the literature (Ault & Stoner, 1993), a mutation (G → A) was frequently observed at nucleotide 108 in the C region.

**Discussion**

In the present study, we have analysed the TCR sequences of the human polyomavirus JCV strains detected in CSF and PBL specimens from individuals with different clinical conditions in order to clarify how JCV spreads within the host and the role of JCV TCR rearrangements.

Twelve out of 15 JCV strains detected in CSF showed TCR sequences with rearranged patterns. The detection of 12 strains with rearranged TCR in CSF was coincident with the diagnosis of PML in the same patients. In three cases, the archetypal TCR structure was demonstrated. This finding was rather unexpected because the presence of JCV with archetypal TCR in the brain has been rarely and only recently described: in one out of five non-PML brain tissues (Elsner & Dörries, 1998) and in the brain tissue of a child who died of PML (Newman & Frisque, 1997) where heterogeneous TCR variants, including the archetype, were present. In our study, one out of the three archetypal JCV strains was obtained from a non-HIV patient with tuberculous meningitis. The two others were from two AIDS patients with neurological disorders which, at the beginning, could be consistent with PML, but later were diagnosed for AIDS-dementia-complex (ADC) and neurotoxoplasmosis, respectively. In the literature, the detection of JCV DNA in CSF samples from two out of eight patients without PML has been reported but, in those cases, the TCR type was not assessed (Matsiota-Bernard et al., 1997). At variance with this observation, in our cases, a presumptive diagnosis of PML was done initially on the basis of clinical findings. Could the detection of JCV with archetypal TCR in their CSF suggest an underlying PML or could the JCV have been passively transported by lymphocytes to the brain and the CSF? In the first case, a high replication activity of the archetype in glial cells should be assumed. Although there are no conclusive elements to exclude this possibility (Ault, 1997; Sock et al., 1996), it seems unlikely. In fact, JCV in vitro replication has been shown only in glial cells infected with rearranged strains and not with archetypal JCV. In addition, the high frequency of detection of rearranged TCR, in comparison with archetypal TCR, in brain tissues and in CSF, shown also in this study, could suggest that the rearranged form grows better in brain tissues than the archetypal form (Agostini et al., 1997; Ault & Stoner, 1993; Sugimoto et al., 1998).

Attempts have been made to identify the elements of the JCV promoter/enhancer sequences which are consistently retained, duplicated or deleted in JCV genomes in the brain in order to explain the ability of rearranged strains, rather than archetypal strains, to grow in the brain (Ault & Stoner, 1993; Agostini et al., 1997). Those studies have shown that rearrangements of JCV TCR are unique but more common patterns exist such as the ‘long duplicate’ pattern in which the majority of our strains, either from CSF or PBL, is also included. It is likely that this rearrangement of the TCR has a higher probability of occurring and/or confers to the virus a better adaptation to grow in glial cells.

Recently, based on the pattern of the regions A–F, the existence of a few JCV TCR variants within the two TCR types, I and II (W1, corresponding to Mad-1, W2 and W4 in type I and W3, W5 and W6 in type II) has been suggested. A variant, W7, corresponded to the archetypal TCR (Elsner & Dörries, 1998). In our study, both JCV TCR type I and type II, in addition to the archetype, have been detected. As regards the variants, only W1 (Mad-1) and W4 were detected in some of our samples; as the results of Elsner & Dörries (1998) also show, in our samples the W4 variant was detected always together with W1 (Mad-1). It is possible that W4 is a defective variant or a precursor of Mad-1. However, most of the TCR described here showed a pattern that was different from that of the other variants described by Elsner & Dörries (1998), either of JCV TCR type I or type II.

The lymphotropism of JCV is now well-documented (Dörries et al., 1994; Tornatore et al., 1992; Azzi et al., 1996; Monaco et al., 1996; Dubois et al., 1997; Gallia et al., 1997). However, the JCV TCR type detectable in lymphocytes has not yet been greatly investigated. To date, rearranged TCR have been reported in lymphocytes from two PML patients (Tornatore et al., 1992) but also from seven patients without PML (Dörries et al., 1994; Tornatore et al., 1992). The results of our previous study (Azzi et al., 1996) by using RFLP analysis suggested that both archetypal and rearranged TCR are detectable in PBL. The data reported here indicate that JCV with rearranged TCR are present in PBL from PML patients. On the other hand, in PBL from individuals without PML, either immunocompetent or immunocompromised, archetypal TCR are present. In addition, a high similarity has been shown in this study between the TCR structure detected in PBL and in CSF from the same patient. This observation, if confirmed by the analyses of more paired PBL and CSF samples, could strengthen the hypothesis that JCV gains access to the brain tissue via infected lymphocytes.

The detection of JCV mRNAs for VP1 capsid protein, recently reported in PBL from one PML patient (Dubois et al., 1997), and the detection of various JCV TCR in the same lymphocyte sample reported in this study suggest that JCV can replicate in lymphocytes in vivo. On the other hand, cellular factors required for JCV proliferation have been shown in lymphocytes (Riedmann et al., 1994). The observed TCR rearrangements may therefore occur as a consequence of active replication.
In conclusion, the data shown in this study indicate that JCV either with rearranged or archetypal TCR infects lymphocytes and suggest that, in lymphocytes, virus replication may also occur. Lymphocytes carry either the archetypal or the rearranged form of the virus to the brain. Here, the virus may persist in latent form and may be reactivated under immunosuppressive conditions or as a consequence of different factors such as inflammation and cytokine production. However, archetypal forms, in contrast with the rearranged forms, are incapable of high replication rates in the brain, a condition that is not consistent with the demyelination characteristic of PML. In such cases, the virus load should be very low. The extreme sensitivity of the assays developed in the last few years to detect viral DNA, mostly based on PCR, allows detection of even minimal amounts of JCV genomes (Dörries et al., 1998) that are unable to cause PML.

From the literature (Matsiota-Bernard et al., 1997; Moret et al., 1993; Sugimoto et al., 1998) as well as from our data, the PCR method for the detection of JCV DNA in CSF samples appears to be undoubtedly useful for the diagnosis of PML. However, because of possible asymptomatic reactivation of JCV in the brain, further studies are required to assess the usefulness of other assays to use in combination with PCR such as quantitative PCR or TCR typing or even assays for anti-JCV antibody titration in CSF (Sindic et al., 1997).

Our results suggest that there is also an association between the detection of rearranged TCR in PBL and PML samples. Because of the contrasting data from the literature, however, further studies will be required to assess the diagnostic and prognostic significance of the detection of rearranged TCR in PBL.

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