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Capsid protein VP1 deletions in JC virus from two AIDS patients with progressive multifocal leukoencephalopathy

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PCR on 52 cerebrospinal fluid (CSF) specimens and 33 brain biopsies obtained from HIV-1 positive patients utilized pairs of primers from both the early region (JTP) and late region (JLP). In these patients, in whom progressive multifocal leukoencephalopathy (PML) was suspected on the basis of clinical symptons and magnetic resonance imaging (MRI) studies, eight CSFs (15%) and 14 brain biopsy specimens (42%) contained JCV DNA sequences. In two patients' samples, the CSFs were positive for JCV DNA in the VP1 region using the primer pair for the VP1 region (JLP), but the fragment amplified migrated more rapidly than the 129-bp product obtained from prototype JCV(Mad-1) or the fragment amplified from the antigenic variant of JCV known as Mad-11. These patients died 3–4 months after onset of progressive neurological symptoms. Cycle sequencing of the fragments revealed overlapping deletions of 24 and 27 nucleotides. These strains were of different genotypes, designated strain 107 and strain 206. Computer analysis of the VP1 amino acid sequence predicts that the eight or nine amino acid residue deletions represent a surface loop with a high antigenic index. These naturally occurring deletion mutants are the first examples of a phenomenon observed experimentally in the mouse polyoma virus capsid protein VP2.

Keywords: cerebrospinal fluid; peptide structure; polymerase chain reaction; polyomavirus

Introduction

DNA viruses, including the polyomaviruses, are ancient and highly stable. Some may have co-evolved as species-specific viruses during mammalian speciation (Soeda *et al*, 1980). This is in contrast to RNA viruses such as the retroviruses, which mutate rapidly, even within a single host, and may move more readily between species. The polyomavirus JCV presents an interesting contrast to other DNA viruses. While the coding region, which exists in at least two different genotypes (Ault and Stoner, 1992), is highly stable, its regulatory region is rearranged within the host to generate variants that may be more highly neurotropic or neurovirulent (Loeber and Dörries, 1988). At the same time, the regulatory region in the excreted and infectious form of the virus apparently retains a stable archetypal structure (Yogo *et al*, 1990). It would be of importance if these mechanisms of DNA rearrangement, operating in unknown ways within the host, were to generate new, highly virulent mutants which are transmissible.

JC virus (JCV) was first identified through its association with brain tissue in the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML) (Padgett *et al*, 1971). Virus obtained from the kidney and urine has primarily the stable 'archetypal' regulatory region structure (Ault and Stoner, 1994). In contrast, in the PML brain the viral regulatory region has been rearranged after major deletions followed by several DNA duplication events (Ault and Stoner, 1993). It is currently thought that the archetypal regulatory region found in virus excreted from the urine represents the transmissible form of the virus, and that these regulatory region rearrangements may be an important step in generating variants which are better adapted for growth in CNS glial cells.

The relatively high rate of infection of the CNS of AIDS patients with JCV to cause PML (approximately 5%) is not fully explained. Undoubtedly, immunosuppression plays a role, but approximately 25% of PML patients present with PML as the first

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manifestation of AIDS before immunosuppression is otherwise clinically manifest (Berger and Levy, 1993). Transactivation by an HIV-1 product such as Tat is a likely mechanism by which HIV-1 directly promotes JCV reactivation (Chowdhury *et al,* 1990). Another effect of coinfection with HIV-1 may be to influence the pattern of alternate splicing of JCV early region mRNA in infected brain cells (Ishaq and Stoner, 1994).

In this report we describe another source of variation in the JCV genome which may contribute to its neurovirulence in AIDS patients. We examined a total of 85 CSF and brain biopsy samples from 80 different patients who were being evaluated to exclude PML. Two pairs of primers were used which amplify segments in the T-antigen coding region and the VP1 capsid protein coding region. In the VP1 coding region we have identified two JCV mutants which show deletions of 24 or 27 nucleotide pairs. These deletions in JCV from AIDS patients are the first identified in the coding region. Their existence indicates that regulatory regions of human polyomaviruses are not the only locus of deletions sustained while the virus is replicating in PML/AIDS patients. This provides yet another mechanism by which JCV replication might be enhanced in HIV-1(+) patients.

Results

PCR using JTP and JLP sets of primers on 52 CSF specimens and 33 brain biopsies obtained from HIV-1 positive patients suspected of having PML showed that eight CSFs (15%) and 14 biopsy specimens (42%) contained JCV DNA sequences. The products generated with the JTP primers were of the expected length, but in two patients' samples the fragments amplified with JLP primers migrated more rapidly in the gel than expected (Figure 1). These strains with apparent deletions were later desigated 107 (a type 1 strain) and 206 (a type 2 strain). The patient from whom strain 206 was amplified had also been biopsied. The JCV fragment amplified from the brain biopsy showed the same anomalous migration as that from the CSF, again indicating a shorter fragment than predicted (not shown). A known serological variant of JCV, designated Mad-11, was also amplified by PCR with the JLP primers. Unlike strains 107 and 206, Mad-11 revealed a full length product, indistinguishable from that of prototype JCV(Mad-1) (Figure 1).

Cycle sequencing was carried out on the JLP amplified products from strain 107 CSF and the strain 206 biopsy specimen. The sequence revealed overlapping deletions of 24 and 27 nucleotides in

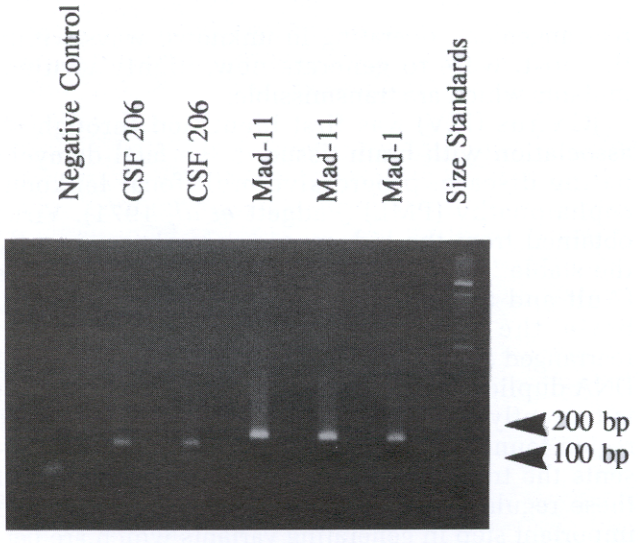

Figure 1 Agarose gel electrophosesis of PCR products amplified with primers JLP-1 and JLP-4. The amplified fragments of strain 107 (not shown) and strain 206 from CSF (duplicate extracts) ran ahead of that obtained from prototype JCV(Mad-1) (129 bp). The JCV (Mad-11) fragment, PCR amplified at two different DNA concentrations, migrates like prototype Mad-1.

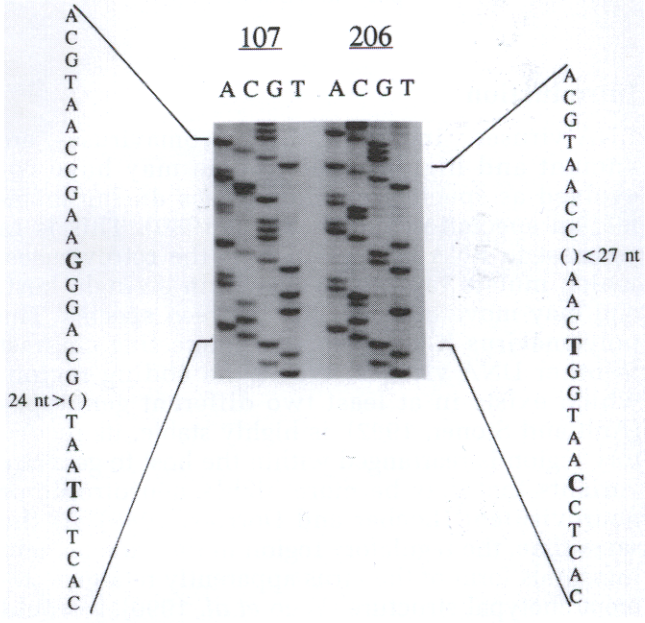

Figure 2 Cycle sequencing of the JLP fragment amplified from strains 107 and 206. Positions of the 24 and 27 nucleotide deletions compared to prototype Type 1 (Frisque *et al,* 1984) and Type 2 viruses (Loeber and Dorries, 1988) are indicated. Likely type-specific sequences are indicated by bold type (see Figure 3). Left: strain 107, sequence of CSF PCR product. Right: strain 206, sequence of brain biopsy PCR product.

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      122                                                          136
Mad-1 His Ser Asn Gly Gln Ala Thr His Asp Asn Gly Ala Gly Lys Pro
     1832                                                         1876
Mad-1 CAC TCT AAT GGG CAA GCA ACT CAT GAC AAT GGT GCA GGG AAG CCA
107   CAC TCT AAT --- --- --- --- --- --- --- --- GCA GGG AAG CCA
206   CAC TCC AAT GGT CAA --- --- --- --- --- --- --- --- --- CCA
GS/B  CAC TCC AAT GGT CAA GCA GCT CAT GAC AAT GGT GCA GGA AAG CCA

GS/B  His Ser Asn Gly Gln Ala Ala His Asp Asn Gly Ala Gly Lys Pro
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Figure 3 DNA sequence and translated amino acid sequence in the region of loop 125–135. Possible type-specific nucleotides in prototype type 1 (Mad-1) and prototype type 2 (GS/B) strains are indicated by bold type. (See also Iida *et al*, 1993). At the positions where the Mad-1 and GS/B strains differ, strain 107 matches the Mad-1 sequence, and strain 206 matches the GS/B sequence. An amino acid change is predicted in the type 2 VP1 sequence at position 128.

each patient's JCV VP1 region (Figures 2 and 3).

Cycle sequencing of the amplified regulatory region of strain 206 using primers JRR-5 and 6 revealed a rearranged regulatory region with region D deleted (data not shown).

The region of the deleted sequence in VP1 (amino acid residues 125–132 and 127–135) was analyzed with the PEPTIDESTRUCTURE and PLOTSTRUCTURE programs (Figure 4). All indices point to a flexible surface loop in the region of the deletion. This peptide therefore also has a high antigenic potential according to the antigenic index of Jameson and Wolf (1988) and could represent a viral epitope.

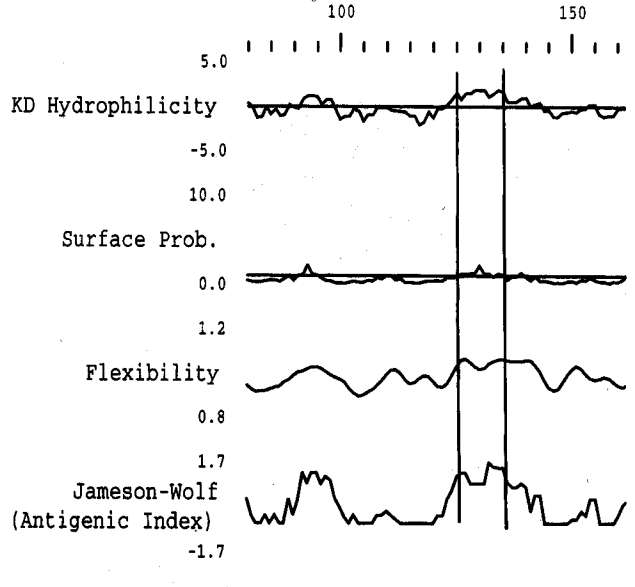

Figure 4 Graphic representation of PEPTIDESTRUCTURE computer analysis of VP1 from residues 80–160. The region of the deletions (residues 125–135) has a high Kyte-Doolittle hydrophilicity and a positive surface probability according to the Emini method, along with a high antigenic index.

Discussion

We have identified two new variants of JCV in AIDS patients which have a deletion of 24 or 27 nucleotides in the late region coding for VP1. These deletions apparently remove a surface loop, and thus do not disrupt the overall structure of the protein. This adds a new level of viral variation to the regulatory region rearrangements (Ault and Stoner, 1993) and coding region types (Ault and Stoner, 1992) which have previously been identified in PML brains. These findings raise several important questions.

Are these new JCV mutants capable of more rapid DNA replication and thus likely to be more virulent than prototype strains?

It can be postulated that these mutants have a selective growth advantage at some level. Otherwise, they would not have become the dominant variant of the virus within the CNS of these patients. Both patients showed a progressive course, with the interval from onset to death being 3–4 months. Whether these strains in fact cause a more widespread CNS disease has not been examined pathologically. Neither has an attempt been made to examine the growth properties of these viruses in cell culture. In the one strain in which the regulatory region could be amplified and sequenced (strain 206), deletion of region D (as previously defined by Ault and Stoner (1993)) was found. This indicates that the VP1 deletion did not occur in place of rearrangement within the regulatory region.

What are the mechanisms by which a capsid protein deletion might enhance polyomavirus replication?

Mechanisms by which capsid deletion might alter viral replication are suggested by recent studies with mouse polyoma virus. In polyoma virus a deletion created in the capsid protein VP2 was found to enhance replication (Melucci-Vigo *et al*, 1994). In

the same study insertions introduced into the polyoma VP2 sequence resulted in defective viruses. It was suggested that the mechanism of replication enhancement involved an encapsidation intermediate whose formation and stability were enhanced by the deletion in VP2. Another model which predicts an effect at the mRNA level, rather than the protein level, is also suggested by studies in mouse polyoma virus. An altered half-life of natural antisense late region mRNA could influence the degradation of complementary early message (Liu *et al*, 1994). Thus, the deletions in VP1 might destabilize naturally occurring antisense transcripts which downregulate early-strand mRNA levels. These two models are not mutually exclusive.

Why is this particular region of the capsid protein VP1 targeted for deletion in two independently arising strains?

We proposed previously that the non-coding regulatory region rearrangements may utilize the chance occurrence of pseudo signal sequences to carry out illegitimate recombination events (Ault and Stoner, 1993). We therefore examined the surrounding VP1 sequence for signals similar to the homologies to the V(D)J joining signals previously identified in the regulatory region (Figure 5). Three copies of the heptamer signal were located between 120 and 151 base pairs downstream from the deletions. One is fully identical to the immunoglobulin signal sequence heptamer (ie CACAGTG); the other two contain the essential first four nucleotides (CACANNN). Only one of the three is followed by a nonamer with the essential dinucleotide base pair, AA, properly positioned with 12 or 23 base pair spacing between hepatmer and nonamer. However, it should be noted that low level recombination can occur in the absence of any nonamer homology (Hesse *et al*, 1989). While this juxtaposition is suggestive, there is no direct evidence that any of these putative JCV heptamer signals is functional.

An antigenic variant of JCV, designated JCV(Mad-11), has been reported to react with anti-JCV antisera to a lesser extent than do other isolates (Padgett and Walker, 1983). It is noteworthy that the Mad-11 strain does not show a deletion in the region of the residue 125–135 loop in VP1 as described here for strains 107 and 206 (Figure 1). If the Mad-11 strain has a capsid protein epitope deleted, it is elsewhere in the VP1 gene or in the VP2/3 gene.

Materials and methods

Cerebrospinal fluid and brain biopsies

CSF or brain biopsies were obtained from patients in whom PML was suspected on the basis of neurological symptoms and MRI findings. The patient's CSF with strain 107 was obtained through the courtesy of Drs Jeffrey Galpin and George Chow. This 50-year-old HIV-1(+) male presented with progressive neurological symptoms, and died 3 months after onset with a diagnosis of PML. The CSF and

Ig consensus signal:		**CACA**GTG---(12/23)--ACAAA**AA**CC
Regulatory region:		
JCV (archetype)		
	(177-204)	**CACA**AGT---(12)-----AGGGG**AA**GT
	(192-219)	**CACA**AGG---(12)-----CAGCC**AA**GG
BKV (WW)	(245-207) (reverse)	**CACA**GGG---(23)-----TAGCC**AA**AC
Coding region:		
JCV (VP1)	(1993-2020)	**CACA**GTG---(12)-----ATGAACACA
	(1993-2031)	**CACA**GTG---(23)-----GCACAAGGC
	(2017-2044)	**CACA**GAG---(12)-----CTAGATAAG
	(2024-2051)	**CACA**AGG---(12)-----AGAAC**AA**AG

Figure 5 Homologies to V(D)J recombination signals in VP1. Essential nucleotides in the heptamer and nonamer of the Ig consensus signal sequence (separated by 12 or 23 base pairs) as described (Hesse *et al*, 1989) are in large bold type. Other viral nucleotides that match the Ig consensus signal sequence are underlined. Putative signal sequences found in the JCV and BKV regulatory regions were previously described (Ault and Stoner, 1993). Numbers in parentheses on the left refer to positions in the archetypal regulatory region of JCV (Yogo *et al*, 1990), the prototype coding region (Frisque *et al*, 1984), or the archetypal WW strain of BKV (Rubinstein *et al*, 1987).

frozen brain biopsy tissue with strain 206 were obtained through the courtesy of Drs Joel Weisman and Scott Binder. The patient, a 39-year-old male, presented with dementia, focal neurological deficits, and generally deteriorating neurological function. MRI showed bifrontal white matter lesions suggestive of PML which was confirmed by histological examination of a brain biopsy. This patient died 4 months after onset of neurological symptoms. No autopsy was done on either patient.

DNA extraction from CSF and tissue
CSF (90 µl) was extracted at 56° for 30 min by addition of 10 µl 0.5% NP40. After centrifugation at 12000 x g for 5 min, the supernatant (10 µl) was used immediately for PCR or frozen. Brain biopsy samples (10–25 mg) were digested overnight at 55° in 100 µl lysis buffer containing 0.2 mg ml^{-1} proteinase K (BRL, Gaithersburgh, MD) and 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM $MgCl_2$, 10% (w/v) gelatin, 0.45% (v/v) detergent NP40 and 0.45% (v/v) Tween20. After boiling for 10 min and centrifugation, 5 µl of the supernatant were used for PCR.

Cloned Mad-11 and Mad-1 DNA
pMad-11-Bra, cloned from the PML brain containing the JCV serological variant originally described by Padgett and Walker (1983) was obtained from RJ Frisque. Cloned JCV prototype strain, pJCV(Mad-1) (ATCC 45027), was obtained from the American Type Culture Collection.

Polymerase chain reaction
Primers JTP and JLP were designed to amplify DNA fragments from the early (T antigen) and late (VP1) coding regions, respectively. Two extracts of each CSF or brain biopsy sample were investigated separately for viral DNA. Based on the prototype sequence for JCV Type 1 (Mad-1 strain) (Frisque *et al*, 1984) [EMBL accession number J02227] and JCV Type 2 (GS/B strain (Loeber and Dörries, 1988) [EMBL accession number M20322], JCV specific primers for both virus types for VP1 were (Mad-1 numbering) JLP-1 (5'CTCATGTGGGAGGCTGT-(G,T) ACCT 3', 1769–1790) with JLP-4 (5'ATGAAA-GCTGGTGCCCTGCACT 3', 1897–1876), and for T antigen JTP-1 (5'GCAGCTTAGTGATTTTCTCAGG 3', 2990–3011) with JTP-2 (5'CACCAAAACAAAA-GAACACAGG 3', 3130–3109). The calculated product lengths were 129 basepairs (bp) for the JLP primers and 141 bp for the JTP primers. Both sets of primers are specific for JCV, and did not amplify BK virus (BKV) or SV40 DNA (data not shown). The regulatory region of strain 206 was amplified from the brain biopsy extract with primers JRR-5 and 6 as previously described (Ault and Stoner, 1993). Samples were added to 100 µl reagent mixture containing standard PCR buffer (Perkin Elmer Cetus, Norwalk, CT) with 1.5 mM Mg^{2+}, 300 nM of each primer and 4.5 U of UITma DNA polymerase with 3'–5' proofreading activity (Perkin Elmer Cetus). dNTPs (200 µM) were added after initial heating at 94°C for 3 min (hot start). The 50-cycle program (Perkin Elmer Cetus, Thermal Cycler 480 or MJ Research, PTC-100) included denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. After a final 10-min extension at 72°C the reaction was stopped at 4°C. Reagent blanks with lysis buffer alone were used as negative controls in each assay. Positive controls included amplification of JCV(Mad-1) as described (Ault *et al*, 1994).

Southern blot
Oligonucleotide probes were JLP-1.1 (5'CTGAGGT-TATAGGGGTGACA 3', 1797–1816) and JTP-1.1 (5'CTGTAAAGTTCTAGGCACTGAATAT 3', 3053–3077) for the JCV VP1 and T antigen PCR products, respectively. Probes were end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and ^{32}P-ATP (Amersham, Arlington Heights, IL). After hybridization of the nylon filter with 1×10^7 cpm of the probe and salmon sperm DNA at 55°C, filters were washed three times with 2 × SSC containing 0.1% SDS. The dry filter was exposed to X-ray film and then developed.

Cycle sequencing
The JLP (strains 107 and 206) and JRR (strain 206 brain only) amplified PCR product were isolated by preparative agarose gel electrophoresis. dsDNA was retrieved by using the GeneCleanII Kit (Bio 101 Inc, Vista, CA). In order to sequence the dsDNA from two directions 100 ng each of the above specified corresponding primers were end labeled with ^{33}P-ATP (Amersham). Radiolabeled primer (1.8 ng) was combined with the cleaned template, DNA polymerase and the chain-terminating dideoxynucleotides (SequiTherm cycle sequencing, Epicentre Technologies, Madison, WI). The initial denaturation for 1 min at 95°C was followed by 30 cycles of 30s at 95°C for denaturation and 1 min at 59°C for annealing and elongation. Products were run on a 6% polyacrylamide gel containing 50% (w/v) urea (National Diagnostics, Atlanta, GA). After fixation the gels were transferred to filter paper, dried under vacuum and developed after exposure to X-ray film for 12–48 h.

JCV typing
JCV strains 107 and 206 were typed by comparison of DNA sequence in the JLP fragment to sequence of prototype Type 1 sequence (Mad-1 strain) (Frisque *et al*, 1984) and prototype Type 2 sequence (GS/B strain) (Loeber and Dörries, 1988). Confirmation was obtained by the typing method previously described (Ault *et al*, 1994).

Peptide structure analysis

Peptide structure and antigenic potential were analyzed using PEPTIDESTRUCTURE and PLOTSTRUCTURE programs from the Wisconsin Package of the Genetics Computer Group (Jameson and Wolf, 1988) on a Silicon Graphics computer.

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