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Analysis of the excreted JC virus strains and their potential oral transmission

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JC virus (JCV) particles have been detected in urban sewage of divergent geographical areas. In this study, the authors evaluate the genetic characteristics and the infective capabilities of JCV strains in relation to the potential oral transmission of JCV in the population. JCV strains excreted in urine and detected in sewage have been described as presenting archetypal structure of the regulatory region of the viral genome. The regulatory region of JCV viral particles detected in two urban sewage samples have been cloned and characterized. From a total of 40 clones tested, 39 presented archetypal-like regulatory regions, whereas 1 of the clones analyzed presented a tandem repeated structure. Archetypal strains present in the urine of a pregnant woman were able to infect SVG cells, producing infectious virions, as demonstrated by confirmative cell culture, electron microscopy, and *in situ* DNA hybridization. This is the first description of archetypal JCV productive infection of SVG cells. SVG cells were also successfully infected with Mad-4 JCV viral particles subjected to pH 3 for 1 h at 37°C and to 10 μg/ml of trypsin in the same conditions. A decrease in the viral progeny production was observed when Mad-4 was subjected to acidic pH. Mad-4 did not produce any detectable infection in the enteric cell line CaCo-2. The oral route could represent a significant route of transmission of JCV infections because JCV virions have demonstrated relative resistance in the environment and to some of the conditions present in the gastrointestinal tract. The archetypal strains commonly detected in the environment may be implicated in the transmission of JCV among the population. Sporadic infection with strains presenting tandem repeated structures may have implications in pathogenicity. *Journal of NeuroVirology* (2003) **9,** 498–507.

Keywords: archetype; environment; gastrointestinal route; infectivity; JC virus; SVG cells

Introduction

JC virus (JCV), one of the members of the Polyomaviridae family and the etiologic agent of progressive multifocal leukoencephalopathy (PML), infects a high percentage of the human population, producing persistent infections in the kidney (Shah, 1995). The infection in childhood is usually asymptomatic, leading to the production of specific antibodies (Taguchi *et al*, 1982). The virus has also been detected in the lung and liver (Grinnell *et al*, 1983), in gastrointestinal tissues (Laghi *et al*, 1999), in lymphoid cells of PML patients (Houff *et al*, 1988), and in peripheral blood leukocytes of non-PML acquired immunodeficiency syndrome (AIDS) patients (Dorries *et al*,

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1994; Tornatore *et al*, 1994; Dubois *et al*, 1996, 1997). When JCV was found in lymphoid preparations after bone marrow transplantation, Schneider and Dorries (1993) suggested the lymphoid tissue as another site of viral latency, with the lymphocytes implicated for trafficking the viruses throughout the body. JCV lytically infects oligodendrocytes, causing PML, a fatal demyelinating disease that affects immunocompromised people, primarily those affected by AIDS. Aproximately 4% of AIDS patients develop PML (Berger *et al*, 1987).

The development of brain tumors in experimental animals has been described (ZuRhein, 1983, 1987; Krynska *et al*, 1999) and JCV has also been reported to transform cells in culture (Theile and Grabowski, 1990). A role for JCV in brain tumors has been recently suggested (Rencic *et al*, 1996; Boldorini *et al*, 1998; Khalili *et al*, 1999; Del Valle *et al*, 1999; Imperiale, 2000; Calderelli-Stefano *et al*, 2000). Although there is no direct evidence for tumorigenesis of JCV in the human central nervous system (CNS), as some articles had reported (Herbarth *et al*, 1998; Del Valle *et al*, 2001), Del Valle *et al* (2001) have recently suggested reevaluating the role of JCV in the pathogenesis of human brain tumors after detecting JCV DNA in 69% of brain tumors analyzed. JCV has also been related to human colorectal cancer (Laghi *et al*, 1999; Ricciardiello *et al*, 2000) and to chromosomal instability in the denominated rogue cells (Neel *et al*, 1996).

More than a 80% of the adult human population present antibodies against JCV (Padget and Walker, 1973). The virus persists in the kidney and is excreted in urine, indicating that the kidney is a site of virus multiplication. Viruses excreted in urine present an archetypal regulatory region (RR) characterized by presenting the 23-bp and 66-bp sections (Yogo *et al*, 1990), usually deleted in the strains present in the brain and cerebrospinal fluid of PML patients, that commonly present tandem-repeated structures in the RR. It is thought that these hypervariable forms are derived from the archetypal type (Ault and Stoner, 1993; Iida *et al*, 1993). The rearrangements occurring in tandem-repeated structures have inferred that JCV has the capability of infecting cell culture with extended cell tropism (Yogo *et al*, 1990, 1991; Iida *et al*, 1993).

Human primary fetal glial cell cultures (PFGCs) efficiently support the growth of JCV presenting tandem-repeated structures in the RR (Padget and Walker, 1971). Hara *et al* (1998) reported the growth of archetypal JCV after infection of COS-7 cells with urine. To our knowledge, there have not been any more descriptions of successful replication of archetypal JCV after infecting cell culture. The route of infection of JCV has not been defined.

In a previous study, we described the detection of high concentrations of JCV in sewage samples and suggested the potential transmission of JCV through the oral route. We described that the viruses excreted presented the archetypal structure in their RR (Bofill-Mas *et al*, 2000). The JC viral particles detected were significantly stable in sewage samples (T_{90} at 26.7 days) and relatively stable to acidic pH treatment (Bofill-Mas *et al*, 2001). In the present study, we have evaluated the infectivity in SVG cells of Mad-4 JCV viral particles treated with acidic pH and with trypsin. We also have studied the capability of infection of JCV archetypal strains from urine and the characteristics of the RR present in urban sewage and potential transmission to the population.

Results

Cloning of the RR of JCV strains detected in sewage sample containing JCV

In previous studies, direct sequencing of the bands obtained by nested polymerase chain reaction (PCR) revealed that the most abundant type of JCV strains excreted presented the archetypal structure of the RR (Bofill-Mas *et al*, 2000, 2001). In the present study, we have cloned the RR of the JCV from sewage samples in two different geographical areas in order to evaluate the diversity of JCV strains present in urban sewage that would potentially infect the population. After sequencing 40 recombinant clones obtained by cloning the amplicons of the RR of the two samples, we determined that 17/20 clones from the Barcelona (Spain) sample were identical in the 235 nucleotides analyzed and identical also in the archetypal RR structure. In 3/20 clones, we observed differences in the archetypal structure. Clone BCN2.32 presented a deletion of one A at the A_8 tract, as previously reported in JCV clones generated from colon cancer samples (Ricciardiello *et al*, 2001). Clone BCN2.5 presented a tandem duplication of a 33-nucleotide sequence (nucleotides 210 to 242 and 243 to 275) in block *f* (Ault and Stoner, 1993) of the RR as previously described (Jensen and Major, 2001, and articles cited there). Clone BCN2.27 presented a single change in nucleotide 181. These variations are summarized in Figure 1**a**.

A total of 12/20 clones obtained from the Washington DC (USA) sample were identical to the archetypal structure. Seven of 20 clones presented little variations, consisting of single changes and small deletions. These variations are represented in Figure 1**b**. Clone USA2.41 presented a tandem-repeated structure of the RR differing in one nucleotide with the sequence described for Mad-4 (Figure 1**c**).

Infection of SVG, COS-7, and CaCo-2 cells with urine samples containing JCV viral particles

In this study, we have assayed the potential infectivity of two urine samples containing JCV viral particles in several cell lines and at long term. The concentration of JCV and BK virus (BKV) present in urine

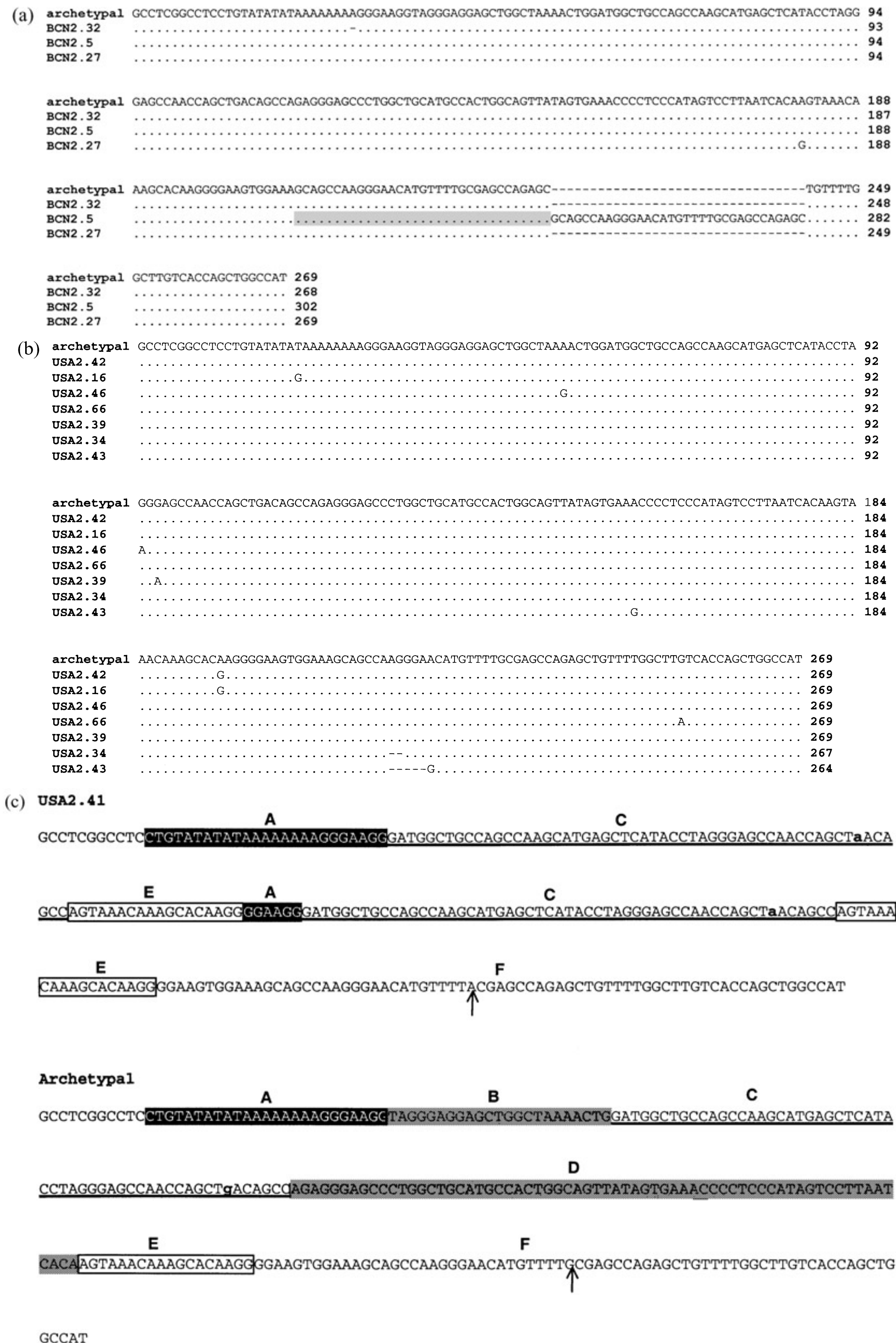

Figure 1 Structure of the regulatory region of archetypal-like clones detected in sewage from Spain (**a**) and USA (**b**) and a tandem repeated structure detected in sewage from USA (**c**). The archetypal conformation is represented in the upper line of the figure. Numbering system is that of Frisque et al. (1984). Dots represent identities while dashes represent deletions. Figure 1**a**, shadowed area represent the nucleotides repeated in the 33bp insertion. Figure 1**c**, from the early side, sections *a* to *f* following the *ori*. Archetypal structure presents sections *b* and *d* while clone USA2.41 present a tandem repeated structure with a partial duplication of section *a* in the second 98 bp repeat. Bold lower case nucleotides represent those typically specific of archetypal or tandem repeated structures. USA2.41 differs one nucleotide with JCV Mad-4, this nucleotide is represented with an arrow and is also different in the archetypal structure.

samples was estimated by limiting-dilution PCR assays. BCNU1 and BCNU2 presented 10^4 JCV viral particles per milliliter, whereas BCNU1 presented also 10^3 BKV viral particles per milliliter. By day 30 post infection, SVG cells infected with urine BCNU1 did not exhibit any obvious cytopathic effect (CPE) or positive result by hemagluttinin (HA) test, when compared with the positive control (Mad-4) that presented clear CPE by day 30 post infection, being positive for HA (1:25,600).

JCV is a cell-associated virus, with small amounts of virus shed into the cell culture medium. These results may also indicate that the quantity of virions, if produced, was too low to be detected by this assay. Stored supernatants were further analyzed by ultracentrifugation, nucleic acid extraction, and PCR. Although negative results were obtained when analyzing supernatants of day 30 post infection, positive results were obtained for days 45 and 70 post infection, with a significant increase of the viral titer between days 45 and 70 as determined by limiting-dilution PCR assays (Figure 2).

The RR and intergenic region (IGR) of the viruses grown in cell culture were sequenced and found to be identical to the sequences previously obtained and reported from JCV present in urine sample BCNU1 (AF119345 and AF120242 in Bofill-Mas *et al*, 2000). An 80-cm^2 SVG flask was infected with 300 μL of the supernatant of day 45 post infection. After observation of CPE at day 15, together with PCR amplification and sequencing of the regulatory and intergenic regions, we confirmed the results previously obtained. Analysis of the supernatants with transmission electron microscopy (EM) demonstrated the presence of polyomavirus-like particles, with a diameter of 37 to 38 nm (Figure 3). These data are consistent with the description of JC and BK virions of 38 to 44 nm diameter reported by Frisque (1986).

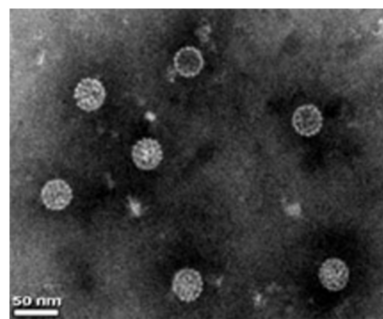

Figure 3 Electron micrograph of JCV present in the supernatant of a SVG cell culture as the result of an infection with a urine sample (BCNU1) containing archetypal JCV. A scale bar (50 nm) and 38 nm virions consistent with polyomaviruses are observed.

Analysis of the supernatant also revealed that archetypal BKV had replicated in SVG cells, although there was one logarithm less of BKV DNA than JCV DNA. The sequence of BKV grown in cell culture was identical to the one obtained from the urine sample. Further confirmation of the infection of SVG cells was obtained by observing positive cells when assaying *in situ* hybridizations of SVG cells grown on cover slides and infected with the supernatant analyzed by EM for 7 days (Figure 4).

COS-7 and CaCo-2 cells infected with BCNU1 did not present any CPE or positive HA or PCR results for a period of 2 months. PCR revealed that Mad-4 positive control had infected COS-7 cells without presenting CPE, whereas there was no evidence of infection of Mad-4 in CaCo-2 cells.

SVG cells infected with BCNU2 did not present any CPE or HA- or PCR-positive results within a period of 3 months. Negative and positive controls corresponded to previous results.

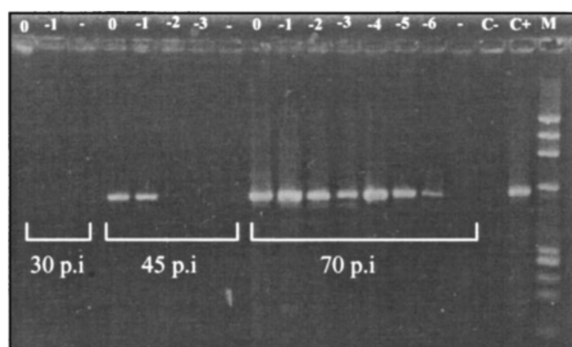

Figure 2 Agarose gel showing the limiting-dilution nested-PCR amplifications of the JCV DNA present in the concentrated supernatants of SVG cell cultures infected with urine sample BCNU1 at different stages of infection (days 30, 45, and 70 post-infection). Negative cell culture controls, PCR negative and positive controls and molecular weight marker (ΦX174 Hae III digested) are also showed. The bands observed (582 bp) are the ones corresponding to the regulatory region of JCV obtained by amplification with JR1 and JR2.

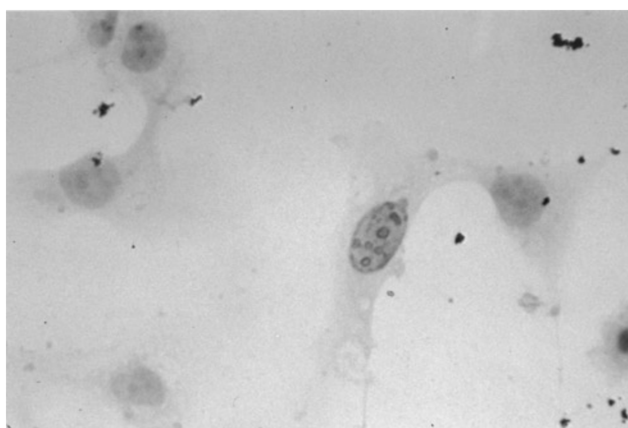

Figure 4 Identification of JC virus DNA in the nuclei of SVG cells infected with viruses isolated from urine of a pregnant woman using *in situ* DNA hybridization. A positive signal is demonstrated by the development of a brown precipitate in the nucleus of the cell that viral DNA is replicating (photomicrograph 40×).

Infection of SVG cells with JCV–Mad-4 stock treated with trypsin and acid pH
As we hypothesized in a previous study (Bofill-Mas *et al*, 2001), the transmission of JCV, which remains still unknown, could occur via the gastrointestinal tract. That would require that viral particles were relatively resistant to proteolytic enzymes and acid pH, conditions present in the gastrointestinal environment. Although archetypal JCV present in a urine sample replicate in SVG cells after 2 months of infection, the stability of JC viral particles at acidic pH or trypsin was evaluated using JCV–Mad-4 strain in order to produce results in experiments with shorter periods of incubation. SVG cells were infected with Mad-4 as a control, Mad-4 subjected to pH 3 for 1 h at 37°C, and Mad-4 subjected to 10 μg/ml of trypsin for 1 h at 37°C. Frozen supernatants from days 3 and 13 were assayed by PCR, obtaining negative results at day 3 for the three stocks assayed and positive results at day 13 for all the infections assayed. The virus concentration in the cell cultures was estimated by PCR limiting-dilution assays. The results were similar in those cells infected with Mad-4 and Mad-4 subjected to trypsin treatment and were 3 logarithms lower in the cells infected with Mad-4 subjected to pH 3 treatment.

Infection of CaCo-2 cells with JCV–Mad-4 stock with and without pretreatment with trypsin
Trypsin has been extensively used as pretreatment for some viruses infecting intestinal cells. We tested the effect of pre activating JCV viral particles with trypsin in the infection of JCV–Mad-4 viral particles in CaCo-2 intestinal cells. Negative results were obtained for CPE and PCR of the cell culture supernatants 45 days after infection. With these results, together with other results obtained in the infection assays in normal conditions described above, we conclude that JCV–Mad-4 did not replicate in CaCo-2 cells under the conditions assayed or after trypsin preactivation.

Discussion

The human gut is frequently exposed to JCV or JCV viral DNA (Bofill-Mas *et al*, 2001). JCV is excreted in urine and found in urban sewage and commonly presents an archetypal structure in the RR (Bofill-Mas *et al*, 2000) as observed by direct sequencing of the JCV isolated in urban sewage.

Some of the studies based on the expression of reporter genes indicated that archetypal JCV exhibited significant activity (Sock *et al*, 1996; Ault, 1997). A study by Hara *et al.* (1998) reported that archetypal JCV produced efficient infection in COS-7 cell culture and described some variations in the sequence of the regulatory region of cultured JCV not observed in the archetypal strains cultured in SVG cells and described in this study. We report in this study that archetypal strains, which are present in the urine of a healthy pregnant woman, have demonstrated infectivity. Pregnancy is the most common condition of altered immunocompetence that has been linked to the activation of polyomaviruses. A low level of neutralizing antibodies in urine may be required for assessing the infectivity of the urine excreted strains. It is known that anti-BKV and anti-JCV neutralizing antibodies are shed in urine and coat the viral particles (reviewed in Knowles, 2001).

We cannot rule out the possibility that coinfection with BK facilitated growth of JCV in SVG cells. However, it should be considered that BK was present in the urine sample in a titre 1 logarithm lower than JC. The estimated quantity of JC and BK viral particles present in 1 ml of urine and used in the infection was of 10^4 and 10^3, respectively. The low number of virus present in the sample reduces the possibility of coinfection of BKV and JCV to a highly improbable event, which is consistent with the higher titter of JCV produced in the infected cell culture in comparison with BKV.

The archetype strains did not show infection in COS-7 or CaCo-2 cells. A review from Jensen and Major (1999) provides an extensive list describing permissive and semipermissive cells that support virus replication. Although a derivative of human PFGCs (e.g., SVG cells) and also tonsillar stromal cells support moderate production of infectious virions after infection with strains presenting tandem-repeated structures in the RR, further studies are required to evaluate the natural cell population responsible for the initial site of infection in humans.

It has been hypothesized that tonsils could be a route of entry. Oral transmission may involve both tonsil and gastrointestinal tissues. Viruses that infect gastrointestinal tissues often multiply in the throat. The results of stability to 10 μg/ml of trypsin and to pH 3 for 1 h also support this hypothesis, although after the treatment with acidic pH, a decrease in the progeny titer was observed. When ingested with food, JCV or JCV DNA may be protected due to the variable pH in the stomach. The ingestion of JCV from contaminated food, water, or fomites could lead to the entry of polyomavirus into the human organism. The digestive tract is also exposed to polyomaviral DNA. Free JCV DNA was detected in sewage treated at pH 1 for 30 min (Bofill-Mas *et al*, 2001). It has been reported that the DNA does not completely degrade in the gastrointestinal tract (Schubbert *et al*, 1997). The oral route of transmission has also been proposed by other authors (Walker *et al*, 1986; Sundsforjd *et al*, 1994; Ricciardiello *et al*, 2000) and, in addition, has also been suggested for the closely related polyomaviruses BKV and simian virus 40 (SV40) (Bofill-Mas and Girones, 2003; Shah *et al*, 1969).

One of the objectives of the present study was to analyze the JCV strains involved in the transmission of infection in the population. The archetypal configuration is highly conserved among JCV strains isolated from urine around the world (Agostini *et al*,

1999). This structure can be modified by both point mutations and small deletions or duplications. Some of these minor changes, found in PML patients, can be related to immunodeficiency or AIDS (Agostini *et al*, 1995; Kitamura *et al*, 1994). Agostini *et al* (1998) reported the finding, in urine, of 7/48 archetypal sequences containing minor changes, such as short deletions, unusual mutations, or atypical duplications. The percentage of sequences containing minor changes correlates with our results. These minor changes have also been reported in urine samples from nonimmunocompromised individuals (Yogo *et al*, 1990) and in immunosupressed renal transplant patients (Yogo *et al*, 1991; Kitamura *et al*, 1994).

The tandem-repeated strain detected in 1/20 clones from a sewage sample collected in Washington differs only one nucleotide with Mad-4.

The Taq polymerase error rate is 1/50 kb and although the possibility of introducing PCR errors in a short fragment during the amplification reaction is very small, this possibility cannot be ruled out. Confirmation of the presence of tandem-repeated strains in the environment will require further studies analyzing higher number of samples.

It has been suggested that tandem-repeated strains evolved from an initial infection of archetypal strains after rearrangement in lymphocytes that produce extended cell tropism; these strains could be related to a higher level of pathogenicity and PML disease after immunossupresion (Yogo *et al*, 2001). We could hypothesize that primary infection would occur through archetypal strains; however, potential implication related to pathogenicity needs to be evaluated for coinfection or reinfection with tandem-repeated strains.

Material and methods

Viruses

JCV–Mad-4 (Padgett and Walker, 1976) viral particles grown on SVG cells that were subsequently partially purified were utilized as a positive control.

Cells

SVG cell line, a cell line established by immortalization of human fetal brain cells with an original defective mutant of SV40 (Major *et al*, 1985), were propagated in Eagle's minimal essential medium (EMEM) supplemented with 1% glutamine, 50 μg/ml of gentamicin, and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated fetal bovine serum (FBS).

COS-7 cell line, a derivative of CV-1, an African green monkey cell line transformed with an origin-defective mutant of SV40 (Gluzman, 1981), was obtained from ATCC. COS-7 were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 1% glutamine, 50 μg/ml of gentamicin, and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS.

CaCo-2 cells, derived from a human colon carcinoma, were propagated in EMEM supplemented with 1% glutamine, 1% nonessential amino acids (NEAA), 50 μg/ml of gentamicin, and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS.

Concentration of viral particles from urine samples

Sample BCNU1 was obtained from a healthy, 38-week-pregnant woman, and sample BCNU2 was obtained from a healthy individual. Cells and viruses present in 120 ml of urine were concentrated by ultracentrifugation for 1 h at 4°C and 229,600 $\times g$ and resuspended in 1 ml of phosphate-buffered saline (PBS). After a nucleic acid extraction (Boom *et al*, 1990), urine samples were tested by nested-PCR, which contained JC (both samples) and BK (BCNU1) viruses as described below. Viruses were resuspended in PBS and stored at −80°C for further experiments.

Concentration of viral particles from sewage samples

A sewage sample (BCN) was collected in the sewers of Barcelona (Spain) in February 2001. The collection in a sterile 500-ml polyethylene container, was kept at 4°C for less than 8 h until the viral particles were concentrated in PBS and stored at −80°C. One sample from the area of Washington DC (USA) was collected in December 1999 and shipped frozen to Spain.

Recovery of viral particles was carried out as described in previous studies (Puig *et al*, 1994; Pina *et al*, 1998). Briefly, 40 ml of sewage sample were ultracentrifuged (229,600 $\times g$ for 1 h at 4°C) to pellet all the viral particles together with any suspended material. The sediment was eluted by mixing it with 4 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min, and the suspended solids were separated by centrifugation at 12,000 $\times g$ for 15 min after the addition of 4 ml of 2 × PBS. Viruses were pelleted by ultracentrifugation (229,600 $\times g$ for 1 h at 4°C), resuspended in 0.1 ml of 1× PBS, and stored at −80°C.

Nucleic acid extraction

Viral nucleic acids were extracted using a procedure that applies guanidinium thiocyanate (GuSCN) and adsorption of the nucleic acids to silica particles (Boom *et al*, 1990), providing clean nucleic acids for genomic amplification.

Enzymatic amplification

Ten-microliter aliquots of the extracted nucleic acids were used in each test. Amplifications were carried out in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$,

Table 1 Oligonucleotide primers used for PCR amplification and sequencing of JCV

Virus type (region)	*Position*	*Primers*	*Reaction*	*AT* [a]	*Sequence*
JCV (IGR)[a]	2062–2087[b]	EP1A	First PCR	59°C	5′-TGAATGTTGGGTTCCTGATCCCACC-3′
JCV (IGR)	2774–2798	EP2A	First PCR	59°C	5′-ACCCATTCTTGACTTTCCTAGAGAG-3′
JCV (RR)[a]	4992–5011	JR1[c]	First PCR	53°C	5′-CCCTATTCAGCACTTTGTCC-3′
JCV (RR)	428–447	JR2[c]	First PCR	53°C	5′-CAAACCACTGTGTCTCTGTC-3′
JCV (RR)	5060–5079	JR3[c]	Nested	53°C	5′-GGGAATTTCCCTGGCCTCCT-3′
JCV (RR)	298–317	JR4[c]	Nested	53°C	5′-ACTTTCACAGAAGCCTTACG-3′
BKV (RR)	5024–5043[d]	BR1	First PCR	44°C	5′-CCCTGTTWARRACTTTATCC-3′[a]
BKV (RR)	431–457	BR2	First PCR	46°C	5′-GTAAAGCAGTGGTACTTT-3′

[a]IGR = intergenic region; RR = regulatory region; AT = annealing temperature; W = A or T; R = A or G.
[b]The sequence positions are referred to the JCV-Mad1 sequence.
[c]From Monaco *et al* (1998).
[d]The sequence positions are referred to the BKV Dunlop strain sequence.

200 μM of each dNTP, 2 units of Ampli Taq DNA polymerase (Perkin-Elmer Cetus), and the corresponding primers at 25 μM. Throughout the PCR assays, the first cycle of denaturalization was carried out for 4 min at 94°C. The conditions for the 29-cycle amplification were denaturing at 92°C for 60 s, annealing at the corresponding annealing temperature (AT) for 60 s, and extension at 72°C for 75 s. All amplifications were completed with a 4-min, 72°C extension period. Primers and ATs used in this study are represented in Table 1. The results were analyzed by agarose gel electrophoresis using ethidium bromide stain.

Standard precautions were applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules.

Sequencing of PCR products
Products obtained after PCR were purified with the QUIAquick PCR purification kit (QIAGEN). Both strands of the purified DNA amplicons were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq DNA polymerase FS (Perkin-Elmer, Applied Biosystems) following the manufacturer's instructions. The conditions for the 25-cycle sequencing amplification were denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. Primers for sequencing were used at 2.5 μM concentration. The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems).

HA assays
HA assays using human type O erythrocytes were carried out as previously described (Neel *et al*, 1996). Briefly, cell culture supernatants were centrifuged for 15 min at 10,000 × *g*. Pellets were resuspended in EMEM 10% DOC (sodium salt deoxycholic acid monohydrate 0.25%) and kept at 37°C for 10 min. Fifty microliters of each sample were serially diluted at 1:2 in Alsever's buffer in a 96-well plate. Fifty microliters of human type O red blood cells (0.5% in Alsever's buffer) were added to each well. Plates were covered and kept at 4°C for 90 min. The HA titer was expressed as the reciprocal of the final dilution which agglutinated the erythrocytes.

In situ *hybridization*
In situ hybridization was carried out as previously described (Askamit, 1985). Briefly, SVG cells grown on coverslips were washed in PBS and fixed for 30 min in 4% paraformaldehyde at room temperature. The cells were dehydrated in serial ethanol washes, hydrolyzed in HCl, washed in Triton-X, subjected to limited protein digestion with pronase, washed in glycine buffer, and dehydrated in serial ethanol washes. The cells were hybridized with 25 μl of a probe mixture containing 10% dextran sulfate, 50% formamide, 0.4 mg/ml calf thymus DNA, 2 μg/ml biotinylated JCV DNA probe (ENZO Biochem), and 2× SSC (300 mM sodium chloride and 30 mM sodium citrate). Probe and cellular DNA were denatured by incubation at 85°C for 10 min. Hybridization was performed at 37°C overnight. Cells were then washed with 2× SSC for 2 min with PBS, then in 0.1% Triton-X and in PBS for 2 and 3 min, respectively. Detection of the biotinylated probe was carried out immediately using streptavidine-biotin–horseradish peroxidase kit (Detek I-hrp, ENZO, Biochem). A fresh solution of diaminobenzidine tetrahydrochloride (DAB) was used as a cromogen. Cells were then washed in 2× SSC, counterstained with hematoxylin, dehydrated, and mounted.

Electron microscopy
Four milliliter of cell culture supernatant were concentrated by ultracentrifugation. Five microliters of the concentrate were deposited on copper grids coated with Formvar film and carbon that were negatively stained with 2% phosphotungstic acid (PTA), pH 7.0, for 1 min. Particles were viewed with a Hitachi H600AB electron microscope.

Cloning of the regulatory region of the JCV present in a sewage sample
Sewage samples collected in Spain and USA were analyzed for the presence of JCV after concentration of the viral particles and nucleic acid extraction. The

primers used in the nested-PCR were JR1, JR2, JR3, and JR4 (Monaco *et al*, 1998) that amplified the RR of JCV. The band obtained after analyzing the results in a 3% agarose gel electrophoresis using ethidium bromide as a stain was directly ligated into a pGEM-T EasyVector (pGEM-T EasyVector System II; Promega) in accordance with the manufacturer's instructions. Ligation was used to transform competent *Escherichia coli* JM109 competent cells (Promega) that were plated on LB plates supplemented with 100 μg/ml ampicilin, 0.5 mM IPTG, and 80 μg/ml X-Gal.

Transformed bacterial clones were detected by blue/white screening. In 20 of the clones obtained from each sample, the insert was recovered by PCR amplification using primers JR3 and JR4. Products obtained after the PCR were further analyzed by sequencing using the amplification primers.

Infection of SVG, COS-7, and CaCo-2 cells with urine samples containing JCV viral particles
Five hundred microliters of concentrated urines BCNU1 and BCNU2 were filtered using 0.22 μm low-protein-binding durapore syringe driven filters (Millex-GV; Millipore) in order to eliminate any bacteria or cells remaining in the urine concentrate.

One hundred microliters of the filtrate BCNU1 were used to infect SVG, CaCo-2, and COS-7 cells plated on 25-cm^2 flasks. Samples were also diluted 1:10 and dilutions were used to infect another set of the three cell types. One hundred microliters of the filtrate BCNU2, and its respective 1:10 dilution, were used to infect SVG cells plated on 25-cm^2 flasks. Negative (100 μl of PBS) and positive controls (100 μl of a 1:1000 dilution of a JCV–Mad-4 stock containing 1024 HA/50 μl) were also assayed. After 1 h at 37°C, maintenance medium was added. Supernatants were renewed weekly using maintainence medium (2% FBS) and stored at −80°C for further studies. After 1 month of culturing, cells were passed at a split ratio 1:2. The time course of the infection was examined by observation of CPE and HA tests. Stored supernatants of different days post infection were ultracentrifugated at 229,600 × *g* for 1 h at 4°C, and pellets were resuspended in 100 μl of PBS. A nucleic acid extraction and a subsequent PCR using primers JR1 and JR2 were carried out. Confirmative cell culture and additional PCR and sequencing of the IGR and RR of the progeny virions were investigated for evidence of infection. Electron microscopy and *in situ* hybridization were also assayed as described above.

Infection of SVG cells with JCV–Mad-4 treated with trypsin and acid pH
A JCV–Mad-4 stock containing approximately 10^5 viral particles per milliliter was aliquoted into three different tubes. Ten μg/ml of trypsin (Gibco) were added to one of the tubes, another tube was treated with diluted HCl until it reached pH 3, and the third tube was kept as a control. Tubes were incubated at 37°C for 60 min. FBS was added to the tube treated with trypsin in order to inactivate the enzyme. The tube treated with HCl was neutralized with diluted NaOH. We infected 25-cm^2 SVG flasks with 100 μl of each of the three stocks. Negatives controls were also assayed. After 1 h at 37°C, maintenance medium was added, renewed periodically, and supernatants frozen for further studies. The time course of the infection was monitored by observation of CPE and PCR of the supernatants.

Infection of CaCo-2 cells with JCV–Mad-4 treated with trypsin
An experiment identical to the one described above was carried out using CaCo-2 cells and Mad-4 viral particles treated with trypsin. The time course of infection was followed by observation of CPE and PCR of the ultracentrifuged supernatants.

Nucleotide sequence accession numbers
Sequences for the Barcelona clones reported in this paper have been deposited in the GenBank database under accession numbers AY083211 to AY083213. Sequences for the Washington DC clones have been deposited under accension numbers AF532778 to AF532779 and AF533308 to AF533313.

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