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Transmission of the Human Polyomavirus JC Virus Occurs both within the Family and Outside the Family

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JC polyomavirus (JCV) is a ubiquitous symbiote in the human population, infecting children asymptomatically and then persisting in renal tissue. We reevaluated the urinary excretion of JCV in subjects in various age groups using PCR. The detection rate for urinary JCV DNA was 9 to 17% until the age of 20 years; this rate increased dramatically to about 46% at the ages of 20 to 29 years and then increased gradually with age. Therefore, it appears that in most people excretion of JCV begins at the age of 20 to 29 years, which is earlier than suggested previously. Next, we studied the way in which JCV is spread in the human population. We selected eight Japanese families in which both parents and children excreted JCV in their urine. Their JCV subtypes were determined by PCR amplification of a JCV DNA fragment; this was followed by restriction enzyme analysis. JCV species in all JCV-positive family members were classified into either of the two subtypes, subtypes CY and MY, which are prevalent in the Japanese population. The following features of JCV subtype distribution were seen in the families: (i) both subtypes were detected in children of five of the eight families, and (ii) of 21 children who excreted JCV, 14 children excreted the same subtypes excreted by their mothers or fathers, while the remainder (7 children) excreted subtypes different from those excreted by their parents. These features suggest that JCV is transmitted both within the family and outside the family. The data also indicate that vertical transmission is not common in the spread of JCV.

Human polyomavirus JC (JCV) is a ubiquitous symbiote in the human population, infecting children asymptomatically and then persisting in renal tissue (4, 18, 20). However, in immunodeficient or immunosuppressed patients, this virus sometimes causes a fatal demyelinating central nervous system disease, progressive multifocal leukoencephalopathy (3, 19).

Although JCV is widespread in the human population (20), its detection has been limited to the brains of patients with progressive multifocal leukoencephalopathy (7, 14, 19), the urine of immunosuppressed patients (see Arthur and Shah [1] for a review), and the urine of pregnant women (5). We have recently shown that older individuals frequently excrete JCV DNA in their urine (10). However, we believed that if a detection method with higher sensitivity were used, urinary JCV DNA could be detected in younger individuals who had been infected with JCV. In the study described here, we reevaluated the urinary excretion of JCV in subjects of various age groups using a much more sensitive method than previously used, that is, PCR. We found that younger adults also frequently excrete JCV DNA in their urine.

Since almost nothing is known about the way in which JCV is transmitted in humans, we attempted to elucidate this phenomenon by tracing JCV in the members of families in which both parents and children excreted JCV in their urine.

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MATERIALS AND METHODS

Urine donors. To elucidate the incidence of urinary JCV excretion in subjects in various age groups, we investigated 239 consecutive outpatients who attended the urology clinics at Branch Hospital, the University of Tokyo, as well as 76 healthy volunteers residing in Tokyo, Japan. Patient conditions were unexplained microscopic and gross hematuria (n = 86), urogenital malignancy (n = 49), urogenital infection (n = 28), urolithiasis (n = 15), benign prostate hyperplasia (n = 14), neurogenic bladder (n = 13), acute and chronic glomerulonephritis (n = 10), hemospermia (n = 7), and others (n = 17). None of the patients were receiving immunosuppressive or anticancer drug therapy.

Eight families were studied to elucidate the mode of transmission of JCV. These families resided in Ishikawa Prefecture (six families) and Tokyo (two families), Japan. The children of these families lived together with their parents throughout childhood, but by the age of about 30 years most of them had married and moved out (see the pedigree in Fig. 2, which shows the ages and genders of the family members).

Extraction of DNA from urine. DNA was extracted from the virion fraction of urine as described previously (10). In brief, after the removal of urinary sediment, urine samples were subjected to high-speed centrifugation at 100,000 × g for 3 h to pellet the virions. DNA was extracted from the resultant pellets by digestion with proteinase K and subsequent phenol treatment. The DNA was then concentrated by ethanol precipitation.

Amplification by PCR. A fragment containing the JCV regulatory region was amplified as described previously (22), with one modification: the reaction was carried out for 50 cycles. The primers used were 5′-GCAAAAAAGGGAAAAA CAAGGG-3′ (JC-1L) and 5′-CATCTGCAGCTGGTGACA

AGCCAAAACAG-3′ (JC-1R), whose locations have been shown previously (22). Primer JC-1R contains a 5′-terminal hexanucleotide, CATCTG, which is not present in JCV DNA. The addition of this sequence generates a *Pst*I cleavage site, CTGCAG. This cleavage site, together with the *Hin*dIII cleavage site, located to the early side of the origin of replication, allows the excision and cloning of the JCV regulatory region from the amplified fragments (see below). We also amplified the "V-T intergenic region" as described previously by Ault and Stoner (2) (data not shown).

After amplification, 5 μl of each reaction mixture was analyzed by electrophoresis on a 1.8% agarose gel containing ethidium bromide (0.5 μg/μl) in TBE buffer (16). The gel was photographed on a UV light transilluminator. The DNA in the gel was blotted onto a nitrocellulose filter with a vacuum blotting system (Pharmacia LKB Biotechnology AB). The filter was hybridized with ^{32}P-labeled JCV DNA and washed as described previously (10).

In preparing template DNA as well as in performing PCR amplification, we took all precautions required to avoid cross contamination (12).

Molecular cloning. PCR-amplified fragments were digested with a combination of *Hin*dIII and *Pst*I, which excises a fragment encompassing the origin of DNA replication to the primer JC-1R-binding site. The recovered fragment was ligated to *Hin*dIII- and *Pst*I-digested, alkaline phosphatase-treated pUC19 and was used to transform *Escherichia coli* HB101 competent cells (Takara Shuzo, Co. Ltd., Kyoto, Japan). Forty-eight plasmids were prepared in miniscale and were used for restriction enzyme analysis.

Restriction enzyme analysis. Restriction enzymes were obtained from Toyobo Co., Ltd., Osaka, Japan (*Hin*dIII, *Pst*I, and *Pvu*II) and Takara Shuzo Co. Ltd., Kyoto, Japan (*Eco*T14I). Digestion with each enzyme was carried out as recommended by the suppliers. Double digestion with *Hin*dIII and *Pst*I was performed at 37°C in medium buffer (16). The digested DNA was analyzed by agarose gel electrophoresis as described above.

Sequencing. Recombinant plasmids containing the JCV regulatory region (the *Hin*dIII-*Pst*I fragment) were sequenced by using an AutoCycler Sequencing Kit and A.L.F. DNA Sequencer II (Pharmacia LKB Biotechnology AB). Sequencing was carried out with overlapping clones representing both DNA strands.

RESULTS

Incidence of urinary JCV DNA in subjects in various age groups. The JCV DNA detection method involving PCR (22) was modified in two ways. The amplification reaction was performed for 50 rather than 25 cycles, and the specificity of amplification was confirmed by blot hybridization. The former modification increased the detection sensitivity to 1 fg of JCV DNA per ml of urine. The latter was helpful for excluding false-positive results, since upon amplification DNA samples containing cellular DNA sometimes generate a trace amount of a fragment whose size is similar to that of the JCV-specific fragment.

Because the sensitivity of detection was greatly increased, it is critical to confirm that the amplified fragments were not generated because of cross contamination. However, as described below, cloning of typical amplified fragments indicated that they were essentially homogeneous. This argues that cross contamination of JCV DNA among samples was so low, if there was any, that it did not influence the detection of JCV DNA in the present study.

TABLE 1. Detection rate for urinary JCV excretion in subjects of various age groups

Age group (yr)[a]	No. (%) of JCV-positive individuals
0–9	3 (8.6)
10–19	6 (17.1)
20–29	16 (45.7)[b]
30–39	20 (57.1)
40–49	19 (54.3)
50–59	21 (60.0)
60–69	22 (62.9)
70–79	22 (62.9)
80–89	23 (65.7)
Total	152 (48.3)

[a] There were 35 individuals in each age group, for a total of 315 individuals examined.
[b] $P < 0.05$ versus 10- to 19-year-old age group.

Thirty-five individuals in each age group (Table 1) were examined for the presence of urinary JCV DNA. JCV DNA was detected in the urine of 152 of the 315 individuals examined (48.3%). This detection rate was markedly higher than that obtained previously by blot hybridization (29.2%) (10).

The incidence of urinary JCV DNA excretion was low (9 to 17%) in the 0- to 9-year-old and 10- to 19-year-old age groups, but dramatically increased, to 46%, in the 20- to 29-year-old age group and then gradually increased with age. The difference in this incidence in the two groups (10 to 19 years and 20 to 29 years), analyzed by the χ^2 test, was significant ($P < 0.05$).

Data were also analyzed on the basis of the patients' conditions. The detection rate for JCV DNA was higher in those with benign prostate hypertrophy (78%), those with malignancy (53%), and those with neurogenic bladder (60%). We interpreted this high detection rate as a reflection of the higher mean ages of the subjects in these patient groups (data not shown).

Distribution of JCV subtypes within families. Most of the JCV strains in the Japanese population belong to one of two subtypes, tentatively named CY and MY (22, 23). These can be differentiated by nucleotide differences distributed throughout the JCV genome (9, 21). For example, in the regulatory region, three nucleotides, at nucleotides 107, 159, and 217, differ in CY and MY. Two of these (nucleotides 107 and 217) are located within restriction enzyme (*Pvu*II and *Eco*T14I) cleavage sites (21). Therefore, the subtypes of Japanese JCV strains can readily be determined by PCR amplification of the JCV regulatory region and subsequent restriction analysis.

We selected eight families in which at least one parent, as well as some children, excreted JCV in their urine. Amplified regulatory regions derived from JCV-positive family members were digested with *Pvu*II and *Eco*T14I and analyzed on a 1.8% agarose gel. Results for a typical example (family 8) are shown in Fig. 1. The mother (lanes 1 and 5) and the older sister (lanes 2 and 6) excreted the MY subtype, while the younger sister (lanes 3 and 7) and the younger brother (lanes 4 and 8) excreted the CY subtype. (The older brother did not excrete JCV DNA.) The detection and subtyping of JCV excreted by the members of the eight families are shown in the pedigrees in Fig. 2.

We also amplified the V-T intergenic region (2) from the family members described above. This region contains a nucleotide variation between CY and MY (position 2251) that

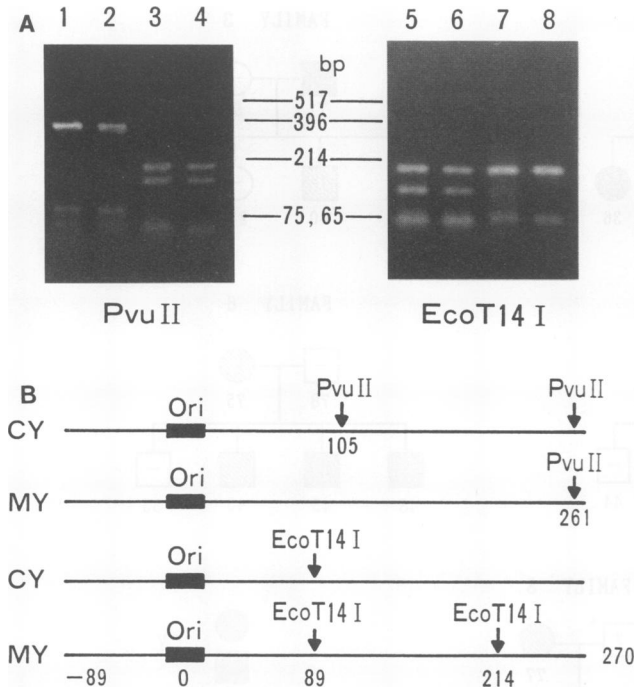

FIG. 1. (A) Classification of urinary JCV into two subtypes, CY and MY, on the basis of restriction fragment length polymorphisms. Fragments containing the JCV regulatory region were amplified by PCR from the urine samples of members of family 8 as described in Materials and Methods. The amplified fragments were digested with *Pvu*II (lanes 1 through 4) and *Eco*T14I (lanes 5 through 8) and analyzed by electrophoresis on a 1.8% agarose gel. Lanes 1 and 5, mother; lanes 2 and 6, older sister; lanes 3 and 7, younger sister; lanes 4 and 8, younger brother; pUC19 digested with *Hin*fI (the sizes of the pUC19-*Hin*fI fragments are indicated in base pairs in the center). No JCV DNA was amplified from the older brother. Lanes 1, 2, 5, and 6 represent subtype MY, while lanes 3, 4, 7, and 8 indicate subtype CY. (B) Restriction enzyme cleavage site maps showing *Pvu*II and *Eco*T14I cleavage sites on the regulatory regions amplified from subtypes CY and MY. The positions of the cleavage sites and the terminal nucleotides of the amplified fragments are indicated by nucleotide numbers. The nucleotide numbering system used is similar to that of Frisque et al. (8), except that positions to the left of the origin of replication (Ori) are shown with negative numbers.

affects cleavability with *Nsp*I (9). Analysis of the amplified fragments with *Nsp*I (data not shown) confirmed the subtyping of JCVs on the basis of the restriction site polymorphisms within the regulatory region.

The following features of JCV subtype distribution were seen in the families (Fig. 2). (i) Both subtypes (CY and MY) were detected from the children of five of the eight families. (ii) In total, 21 children excreted JCV; 14 excreted the same subtypes excreted by their mothers or fathers and 7 excreted subtypes different from those excreted by their parents. (iii) In two families (families 3 and 5), although the mothers did not excrete JCV, 50% or more of their children did so. (iv) In six families, mothers excreted one JCV subtype (MY), while 50% of their children (n = 8) excreted this subtype and the other 50% (n = 8) excreted the other subtype (CY). These features suggest that JCV is transmitted both within the family and outside the family. The data also indicate that vertical transmission is not common in the spread of JCV.

Homogeneity of JCV species in family members. To elucidate whether superinfection with JCV occurred in the study population, we analyzed the homogeneity of excreted JCV. Nine family members, all children (Fig. 2), were selected because their siblings excreted a JCV subtype different from the one that they excreted. Regulatory region fragments amplified from JCV in the urine of these subjects were molecularly cloned. Thirty-seven to 47 recombinant clones containing the JCV regulatory region were examined for subtypes by restriction analysis by using *Pvu*II and *Eco*T14I as described above (Table 2).

Although the subtypes of most clones were unequivocally determined with both restriction enzymes, the subtypes of rare clones were not so determined (Table 2). We determined the entire nucleotide sequences of the rare clones (eight in all) whose subtypes remained uncertain after restriction analyses. Their subtypes were then determined on the basis of the three nucleotides that differ in CY and MY (see above). Without exception, the subtypes of the rare clones thus determined were consistent with those of the major clones determined by restriction analyses.

Sequencing of rare clones also revealed that, with one exception, they lost the subtype-specific cleavage sites because of mutations other than those at subtype-specific positions (Table 2). These mutations were probably introduced during PCR (15). Thus, it is likely that nucleotide sequence variation other than a subtype-specific one was rare in the regulatory region.

From the data shown in Table 2, we concluded that JCV excreted by the examined family members was essentially homogeneous with respect to JCV subtypes, suggesting that humans are resistant to superinfection with JCV.

DISCUSSION

It has been shown that JCV replicates in the kidney and that the progeny virions are excreted into urine (18). Thus, the excretion of JCV DNA in urine reflects JCV replication in renal tissue. Therefore, our finding that urinary JCV DNA is frequently excreted throughout adulthood suggests that, in most adults, persistent JCV is not latent but replicating.

We showed that the point of conversion from low to high levels of JCV excretion was in the decade from 20 to 29 years of age. The time of this conversion was delayed compared with the time at which the highest rate for the acquisition of anti-JCV antibodies occurred (13, 17) (see below). This delay suggests that a change in the mode of JCV persistence, from latency to replication, occurs at the age of 20 to 29 years. To confirm this idea, we are screening both serum antibodies and urinary JCV simultaneously in individuals of various age groups.

Despite the knowledge that JCV is widespread in the human population (20), almost nothing is known about the way in which JCV is transmitted in humans. In the present study, we addressed this issue and found that JCV is transmitted horizontally, both within the family and outside the family.

Coleman et al. (5) and Daniel et al. (6) investigated whether JCV is congenitally transmitted to the fetus. They did not demonstrate immunoglobulin M antibodies against JCV in umbilical cord sera. Taguchi et al. (17) tested antibodies against JCV in the sera of 384 children in Tokyo, Japan. After the maternal antibodies against JCV disappeared in the babies' sera, the children gradually acquired antibodies with age. These serological and seroepidemiological studies, together with our current molecular epidemiological study, exclude a

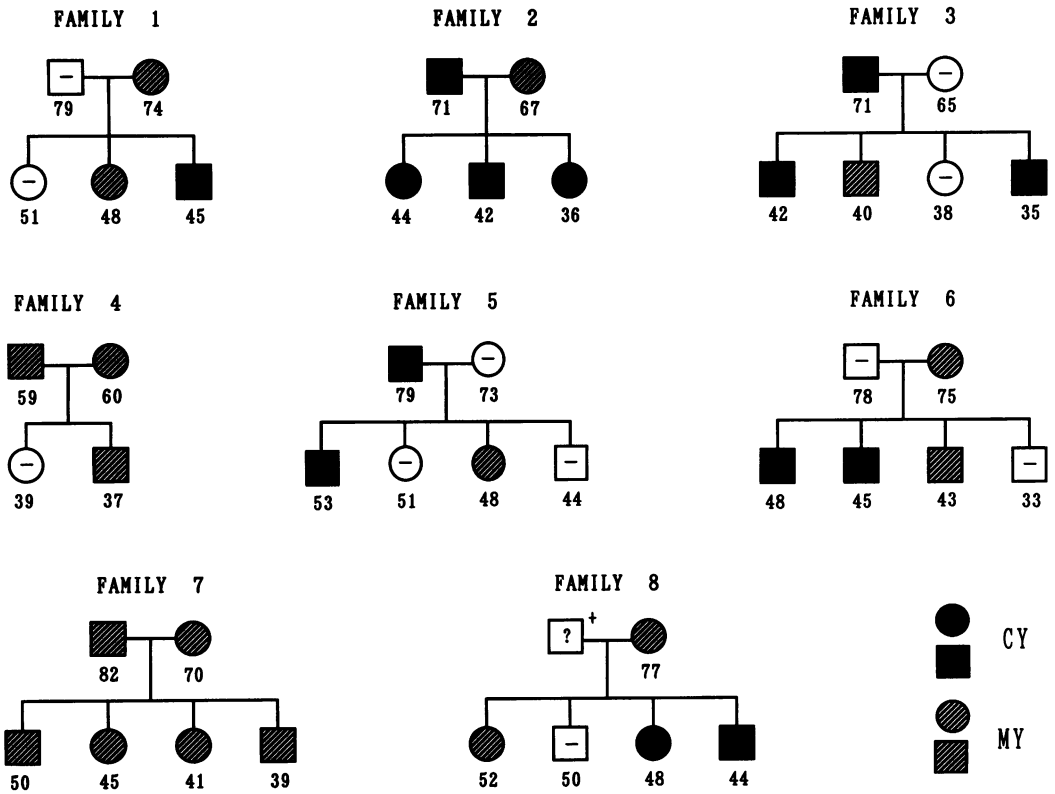

FIG. 2. Pedigrees showing JCV subtypes in the urine excreted by members of eight Japanese families. JCV subtypes were determined as described in Fig. 1. Closed circles and boxes indicate members excreting the CY subtype, and hatched circles and boxes indicate those excreting the MY subtype. The numbers below the boxes denote ages (in years). Families 1 through 6 lived in Ishikawa Prefecture, while families 7 and 8 resided in Tokyo, Japan. Abbreviations: −, not detected; ?, not done.

vertical route for the transmission of JCV, including the transplacental route.

The most probable candidate for the source of JCV in horizontal infection seems to be urinary JCV, since the urinary excretion of JCV in most people starts in early adulthood, and children have many opportunities to come into contact with urinary JCV that may be excreted by their parents, grandparents, babysitters, neighbors, and so on. The finding that siblings in many families had different JCV subtypes may reflect the presence of multiple sources of JCV. Infection with urinary JCV, on the other hand, may be very inefficient, so that freuent contacts with JCV are required for one successful infection.

TABLE 2. Homogeneity of urinary JCV[a]

Children			No. of clones		
Family no.	Gender[b]	Age (yr)	CY	MY	Others
1	M	45	47	0	0
3	M	42	43 (1)[c]	0	1[d]
3	M	40	0	41	0
5	M	53	37 (2)	0	0
5	F	48	0	40 (1)	0
6	M	48	43 (1)	0	0
6	M	43	0	43 (1)	0
8	F	52	0	44 (1)	0
8	F	48	44	0	0

[a] Regulatory region fragments amplified from the indicated children were molecularly cloned with pUC19. Recombinant clones were examined for their subtypes by restriction analysis by using *Pvu*II and *Eco*T14I as described in the text. The entire nucleotide sequences were determined for clones whose subtypes remained unclear after restriction analyses, and their subtypes were determined on the basis of the three nucleotides that differ in CY and MY (see text).

[b] M, male; F, female.

[c] Numbers in parentheses indicate clones whose subtypes were determined on the basis of their sequence data.

[d] One subtype-specific nucleotide (nucleotide 107) was not identical to that of CY or that of MY, while the other two subtype-specific nucleotides were the same as those of CY.

In the nine children analyzed for JCV homogeneity, selected because they excreted a JCV subtype different from that excreted by their siblings, we expected that there would have been many chances for them to have been superinfected with the other JCV subtype. However, in all of these subjects, the JCV subtype excreted in urine was essentially homogeneous. This finding suggests that humans are resistant to superinfection with JCV. In other words, although in humans internal JCV replicates in renal tissue, external JCV does not enter or persist.

Human resistance to JCV superinfection would imply the existence of host immunity to JCV. This immunity may be established after primary infection with JCV. Although this immunity may not be able to eradicate persistent JCV in the host, it may be effective in preventing expansion of the persistent virus. That this is so is demonstrated by findings showing the active growth of JCV during immunosuppression in renal transplant patients (11). This immunity enables the persistent JCV to survive as a symbiote without growing too extensively to kill its host.

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