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Short Communication

Persistence of neurotropic JC virus DNA in hamster tissues six months after intracerebral inoculation

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Immunostaining and polymerase chain reaction (PCR) methods were used to examine tissues from 18 6-month-old hamsters intracerebrally inoculated with JC virus (JCV) as newborns. JCV DNA was detected in all hamster brains and urinary bladders, as well as in most kidney, adrenal gland and pancreas samples. While results from reverse transcription PCR (RNA PCR) and immunostaining suggest that T antigen transcription and protein expression were restricted to the brain, the DNA evidence suggests that intracerebrally inoculated JCV enters the systemic circulation and latently infects organs in a tissue specific manner.

Keywords: JC virus; hamster; latency; polymerase chain reaction

The human polyomavirus JC virus (JCV) causes the fatal central nervous system demyelinating disease, progressive multifocal leukoencephalopathy (PML), which occurs in immunocompromised individuals, especially AIDS patients (Berger *et al*, 1987). In PML, reactivated JCV infects oligodendrocytes and astrocytes (Itoyama *et al*, 1982; Mazlo and Tariska, 1982). While initial infection by JCV occurs during late childhood (Padgett and Walker, 1973), the host cells initially infected as well as those which harbor the latent virus have not been established. The kidney appears to be a primary site of JCV latency, and recent polymerase chain reaction (PCR) evidence suggests that urinary excretion of JCV commonly occurs in healthy individuals beginning in the third decade of life (Kitamura *et al*, 1994; Agostini *et al*, 1995).

In the hamster, JCV induces brain tumor formation when injected intracerebrally (Walker *et al*, 1973; Padgett *et al*, 1977; ZuRhein, 1983) and peripheral neuroblastomas when injected intraocularly (Varakis *et al*, 1978) or intracerebrally (Padgett *et al*, 1977) into newborns. While the oncogenic properties of JCV in the hamster have been the primary focus of studies, this model may also provide valuable information concerning cellular tropism and viral latency. In order to evaluate JCV distribution and potential sites of latency in the hamster, we examined the brains and samples of body organs from 6-month-old hamsters inoculated with JCV as newborns. PCR methods were used to detect the presence of JCV DNA in tissue samples. In addition, transcription and expression of the JCV regulatory protein, large T antigen, were assessed in the brains and urinary organs using reverse transcription (RNA) PCR and immunostaining methods.

Golden Syrian hamsters (Lak:LVG; Charles River Breeding Laboratories) less than 24 h old received a single intracerebral injection of JCV (2500 hemagglutinating units of the MAD-1 strain grown in primary human fetal glial cell cultures). Control hamsters received injections of the supernatant from uninfected primary human fetal glial cell cultures. Six months after JCV inoculation, the hamsters were sacrificed by intracardiac perfusion with sterile phosphate buffered saline (PBS). The brains, kidneys, adrenal glands, spleen, urinary bladder, testis, ovaries, as well as portions of the liver, pancreas and uterine horn were removed using sterile instruments soaked in 0.1% Clorox (15 min) prior to dissection of each organ from each animal. The brain was bisected and one half was immersed in 10% buffered formalin for pathological studies. The opposite half of the brain and other organ samples were frozen by immersion in iced 2-methylbutane. Using separate disposable scalpels to cut each specimen, approximately 10–20 mg of tissue was transferred to sterile 500 μl tubes for PCR analysis and approximately 100–150 mg of tissue was transferred to sterile 5 ml tubes for isolation of mRNA and RNA PCR analysis. The remaining frozen tissue was cryosectioned and immuno-

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stained for detection of JCV T antigen using methods previously described (Ressetar *et al*, 1992, 1993). Additonal sections were stained with hematoxylin and eosin and examined for pathology.

To detect JCV DNA, tissue samples were vortexed and digested for 2 h at 56°C in 100 μl lysis buffer containing 0.2 mg/ml proteinase K (BRL), 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM $MgCl_2$, 10% (w/v) gelatin, 0.45% (v/v) NP40 and 0.45% (v/v) Tween 20. The digests were boiled for 10 min and centrifuged and 5 μl of the supernatant used for PCR. Two tissue samples from each organ were examined by PCR as well as 10-fold and 100-fold dilutions of each tissue extract. Tissue samples were analyzed using two different sets of primers to amplify the T antigen-coding region based on the prototype sequence for JCV (MAD-1 strain) (Frisque *et al*, 1984) [EMBL accession number V01118]. These primers were: JEP-1 (5′-CCTGTGTGTC-TGCACCAGACGC-3′, 2674–2695) with JEP-2 (5′-GGCCAGTTGCTGACTTTGCAGC-3′, 2919–2898) (calculated product length 246 basepairs) and JTP-1 (5′-GCAGCTTAGTGATTTTCTCAGG-3′, 2990–3011) with JTP-2 (5′-CACCAAAACAAAAGAACA-CAGG-3′, 3130–3109) (calculated product length 141 basepairs). Samples were added to 100 μl reagent mixture containing standard PCR buffer (Perkin Elmer Cetus) with 1.5 mM Mg^{2+}, 300 nM of each primer, 4.5 U of UITma DNA polymerase with 3′–5′ proofreading activity and 200 μM dNTPs. The PCR programs (Perkin Elmer Cetus, Thermal cycler 480) 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. When using primers JEP-1 and JEP-2, the program was run for 40 cycles. When using primers JTP-1 and JTP-2, the program was run for 50 cycles with addition of the dNTPs after initial heating of the samples at 94°C for 3 min (hot start) (Stoner and Ryschewitsch, 1995).

JCV mRNA was isolated directly from brain, kidney and bladder samples using the QIAGEN Oligotex™ direct mRNA isolation kit according to manufacturer's instructions. Isolated mRNA was immediately reverse transcribed and the resulting cDNAs amplified using the GeneAmp EZ rTth RNA PCR kit (Perkin Elmer). Intron differential RNA PCR was performed using primers located in different exons of the T antigen gene. These primers used to reverse transribe and amplify large T antigen cDNAs were JEX-1 (5′-TTCTACTAGTATGTATTC-CACCAGGATTCC-3′, 4371–4400) with JEX-2 (5′-C CTT CTCGA GTCT GCATGG GGGAAC ATTCC-3′, 4960–4931) (calculated product length, 246 base-pairs) (Ishaq and Stoner, 1994). Following manufacturer's instructions, 5 μl of mRNA (30–45 ng) was added to a reaction mixture containing standard buffer with 2.5 mM $Mn(OAc)_2$, 100 ng of each primer in 1.5 μl, 0.2 U of rTh DNA polymerase, and 300 μM dNTPs. The PCR program included reverse transcription for 30 min at 70°C, an initial step of 94°C for 2 min, 40 cycles of 94°C (1 min), followed by a final extension for 10 min at 72°C.

For hybridization of the PCR products from the DNA studies, the oligonucleotide probes used were JEP-1.1 (5′-CAGTGCTTGATCCATGTCCAGAGTC-3′, 2731–2755) and JTP-1.1 (5′CTGTAAAGTTCT-AGGCACTGAATAT-3′, 3053–3077). For specific hybridization of the cDNA PCR products from the mRNA studies, the oligonucleotide probe used was LTA-1.1 (5′-TTGGCACCTCTGAACT-3′, 4771–4426) which spans the exon junction (Ishaq and Stoner, 1994). Probes were end-labeled with T4 Polynucleotide Kinase (New England Biolabs) and ^{32}P-ATP (Amersham). 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.

JCV DNA was detected in the brains of all JCV-inoculated hamsters including those which did not exhibit neoplasia (Table 1). Organs found positive for the presence of JCV are summarized in Table 1. JCV DNA was amplified in all samples indicated using both JEP-1 and 2 and JTP-1 and 2 primers (Figure 1). On a comparative basis, brain, kidney and urinary bladder gave strong hybridization signals for JCV DNA while the adrenal gland and pancreas presented weaker signals with amplification increasing slightly upon dilution of the tissue extracts. JCV DNA was amplified in four spleens when the tissue extracts were diluted 1:10 or 1:100. One liver sample and two ovary samples presented very weak hybridization signals only when the tissue extracts were diluted 1:100. Brain and organ samples from control hamsters were consistently negative for the presence of JCV DNA.

JCV induces both adrenal and extraadrenal neuroblastomas when injected intraocularly (Varakis *et al*, 1978) and also has been reported to induce a low incidence of peripheral neuroblastomas when injected intracerebrally (Padgett *et al*, 1977) suggesting that intracranially injected JCV can infect non-CNS tissues. After intracerebral inoculation of JCV into newborn hamsters, the virus enters the brain parenchyma and presumably the cerebrospinal fluid and vascular system, gaining access to the systemic circulation. Our findings of JCV DNA in a high percentage of kidney, bladder adrenal gland and pancreas samples, in addition to the brain, indicates that the virus enters and persists in these tissues for as long as 6 months after inoculation.

In the hamster brain, JCV initially infects mitotic cell populations including granule neurons, subependymal cells, choroid plexus epithelial cells (Ressetar *et al*, 1990) and vascular endothelial cells (Ressetar *et al*, 1992) with JCV DNA passed within a cell population via host cell division. JCV which

Table 1 Tissue samples from 6 month-old JCV-inoculated hamsters found positive (+) or negative (−) for the presence of PCR-amplified JCV DNA

					Organs						
Animal	*Brain*	*Kidney*	*Bladder*	*Adrenal*	*Pancreas*	*Spleen*	*Liver*	*Ovary*	*Uterus*	*Testis*	*Brain pathology*
1	+	+	+	+	+	−	−	−	−		
2	+	+	+	+	+	−	−	−	−		C,S
3	+	+	+	+	+	+	−			−	
4	+	+	+	+	+	−	−	+	−		
5	+	+	+	+	−	−	−	−	−		C
6	+	+	+	+	−	−	−	−	−		C,G,CP
7	+	+	+	+	+	+	−			−	S
8	+	+	+	+	+	+	+			−	C,G
9	+	+	+	+	−	−	−	−	−		S
10	+	+	+	+	−	+	−	−	−		
11	+	+	+	+	+	−	−	−	−		
12	+	+	+	−	+	−	−	−	−		C,G
13	+	+	+	+	+	−	−			−	O
14	+	−	+	+	−	−	−	+	−		C,G
15	+	+	+	−	−	−	−	−			G
16	+	+	+	−	+	−	−			−	
17	+	+	+	+	+	−	−	−	−		C
18	+	+	+	+	−	−	−			−	S
Total	18/18	17/18	18/18	15/18	11/18	4/18	1/18	2/12	2/12	0/6	

C=cerebellar primitive neuroectodermal tumor, G=midbrain glioblastoma, S=subependymal tumor, CP=choroid plexus tumor, O=olfactory bulb tumor

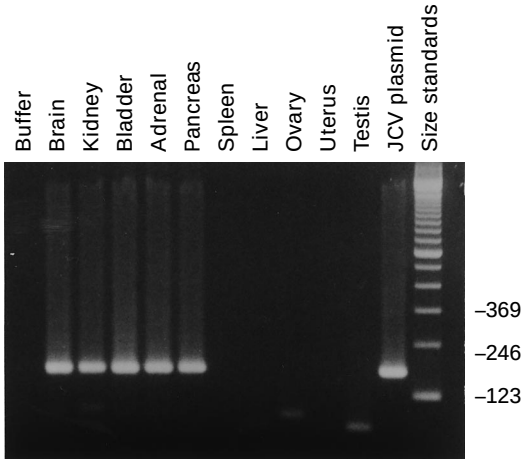

Figure 1 Agarose gel electrophoresis of PCR products from one male hamster (testis) and one female hamster (other organs) amplified with primers JTP-1 and JTP-2. Size standards (bp) are indicated to the right of the gel.

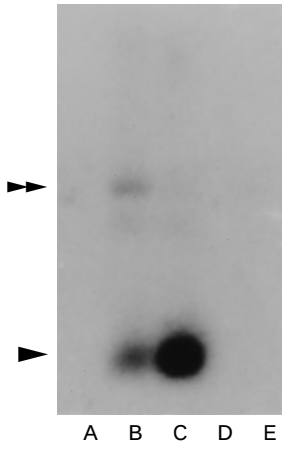

Figure 2 Southern blot analysis of RNA PCR products hybridized with the radiolabeled probe, LTA-1.1, specific for large T antigen. Lanes: A, control hamster brain; B, JCV-inoculated hamster brain not exhibiting neoplasia; C, tumor-bearing JCV-inoculated brain; D, kidney and; E, bladder from JCV-inoculated hamsters. The arrowhead indicates the cDNA band of 246 basepairs. The double arrowheads indicate the heteroduplex consisting of cDNA and genomic DNA PCR fragments formed during the amplification of large T cDNA.

enters the systemic circulation may also infect mitotic stem cell populations. Epithelial cells such as those found in the kidney tubule and duct epithelium, bladder transitional epithelium, and glandular epithelium would provide a pool of cells potentially susceptible to JCV infection and latency.

Adrenal neuroblastomas frequently occur in transgenic mice carrying JCV (Small *et al*, 1986) or JCV-SV40 gene constructs (Feigenbaum *et al*, 1992; Ressetar *et al*, 1993). In these animals, the JCV regulatory region directs the tissue-specific expression of adrenal pathology. Our findings of JCV DNA in the adrenal gland, along with earlier reports of JCV-induced adrenal neuroblastomas in hamsters (Varakis *et al*, 1978) suggest that this gland may be a site of infection and latency in the hamster. While JCV has not been associated with pancreatic neoplasia, another human polyomavirus, BK, induces carcinoma of the pancreatic islet cells when injected i.v. into hamsters (Corallini *et al*, 1978). The high incidence of JCV DNA detected in the

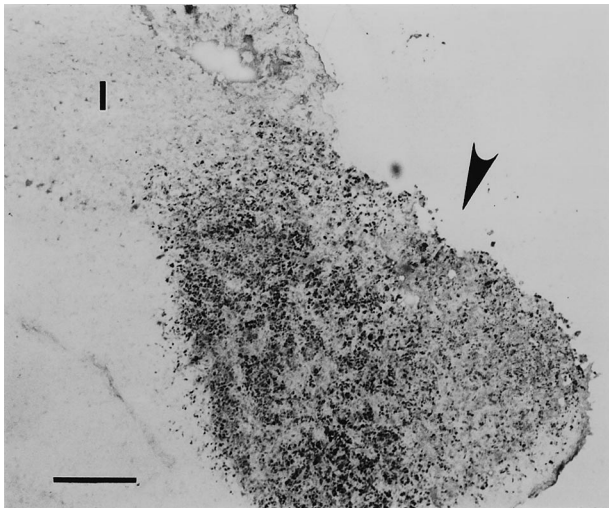

Figure 3 A frozen section of cerebellum from a 6 month-old JCV-inoculated hamster stained with primary antibody (PAb 1614) to T antigen. Arrow indicates a small tumor extending from the internal granular layer (I). Nuclear T antigen expression (black) is pronounced in the majority of the tumor cells. Bar: 20 μm.

pancreas of hamsters in our study suggests that JCV, like BK, may exhibit tropism for cells in this organ.

JCV was detected in the kidneys of all but one inoculated hamster. Since JCV persists in human kidneys, this finding directs interest toward further study of the kidney in the hamster model as a host organ for JCV latency. JCV DNA was present in the bladders of all hamsters. While this finding may be due to actual infection of bladder cells, it may also reflect JCV DNA present in kidney or ureter epithelial cells shed in the urine and retained in the bladder.

Though the hamsters were perfused prior to dissection, other potential sources of the JCV detected in organ samples could be residual blood cells or vascular endothelial cells. Using immunostaining methods, JCV T antigen has been detected in vascular endothelial cells in the brains of hamsters 8 to 31 days after intracerebral inoculation (Ressetar *et al*, 1992) and was also expressed in brain vascular endothelial cells in six of the 6-month-old animals. However, in our PCR analyses, JCV DNA was rarely detected in such highly vascular organs as the liver, spleen, testis, ovary and uterus. This suggests that blood cells are not the source of JCV and if vascular endothelial cells are a source of JCV in the positive organ samples, it is in a tissue-specific manner.

JCV DNA was either not detected or detected in a low incidence in the spleen, liver, testis, ovary and uterus. Our inability to detect JCV in these organs could have been caused by intrinsic tissue factors which inhibited JCV amplification. Tissue inhibitory factors appeared to be present in the spleen where JCV DNA was amplified in four samples after dilution of the tissue extracts. However, even with dilution, JCV DNA was detected in only one liver and two ovary samples. When a 1 : 2000 dilution of JCV-positive hamster brain extract was added to all undiluted extracts of all organs, JCV DNA was amplified in every sample. Amplification of these very low levels of added JCV in liver, ovary, uterus and testis samples suggests that if intrinsic factors present in these organs prevent amplification of JCV DNA, the JCV levels are significantly lower than in other JCV-positive organs.

Large T antigen mRNAs were isolated from the brains of inoculated hamsters (eight of 10 analyzed), including those which did not exhibit neoplasia (Figure 2). However, when frozen brain sections were immunostained with antibodies to JCV T antigen, nuclear T antigen expression was detected only in brains exhibiting neoplasia (Figure 3). In these brains, T antigen was expressed in preneoplastic and neoplastic cells as well as in vascular endothelial cells. These findings suggest that after 6 months detectable levels of T antigen transcription occur in all inoculated hamster brains but translation and expression of protein may be linked with expression of cellular oncogenes involved in neoplastic transformation. Expression of T antigen in vascular endothelial cells was restricted to brains which also exhibited neoplasia (Ressetar *et al*, 1992 and unpublished data) indicating that cellular oncogenes may also be activated in these cells.

The non-CNS organs did not exhibit pathology or T antigen expression on immunostained sections. Detectable levels of T antigen mRNAs could not be isolated from JCV DNA-positive kidney or bladder samples. These findings suggests that in these organs the virus establishes a truly latent infection.

Our findings indicate that significant levels of JCV DNA can be detected in the hamster brain as well as in specific non-CNS organs 6 months after intracerebral inoculation. While JCV T antigen protein expression coincides with brain neoplastic transformation, RNA transcription can also be detected in non-neoplastic brains. JCV which gains access to the systemic circulation infects organs in a tissue specific manner. Though pathology is not evident, the presence of JCV DNA in the hamster kidney, bladder, adrenal gland and pancreas suggests that JCV latently infects cells in these organs.

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