Animal Model

Cynomolgus Polyoma Virus Infection

A New Member of the Polyoma Virus Family Causes Interstitial Nephritis, Ureteritis, and Enteritis in Immunosuppressed Cynomolgus Monkeys

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Polyoma virus infection causes acute interstitial nephritis and ureteral stenosis in humans but has rarely been noted in other species. In the present study, a hitherto unknown polyoma virus was detected in 12 of 57 cynomolgus monkeys after 3 to 11 weeks of immunosuppression given to promote acceptance of renal allografts or xenografts. This virus, termed cynomolgus polyoma virus (CPV), is antigenically and genomically related to simian virus 40 (SV40). The tubular epithelial nuclei of the collecting ducts in the medulla and cortex reacted with an antibody for the SV40 large T antigen and by electron microscopy contained densely packed paracrystalline arrays of 30- to 32-nm diameter viral particles. A polymerase chain reaction analysis of DNA extracted from affected kidneys detected polyoma virus sequences using primers for a highly conserved region of the large T antigen of polyoma virus. Sequence analysis showed 7 base substitutions and 3 to 5 deletions in the 129-nucleotide segment of amplified products, compared with the corresponding portion of SV40, yielding 84% homology at the amino acid level. CPV caused interstitial nephritis in six renal allografts, a xenograft kidney, and six native kidneys. Infected animals showed renal dysfunction and had tubulointerstitial nephritis with nuclear inclusions, apoptosis, and progressive destruction of collecting ducts. CPV was detected in the urothelium of graft ureters, associated with ureteritis and renal infection. Viral infection was demonstrable in smooth muscle cells of the ureteric wall, which showed apoptosis. One animal had diar-

rhea and polyoma virus infection in the smooth muscle cells of the muscularis propria of the intestine. Spontaneous resolution occurred in one case; no animal had virus detected in tissues more than 3 months after transplantation. Thus, immunosuppression predisposes cynomolgus monkeys to a polyoma virus infection with clinical consequences quite similar to BK virus infection in humans, including renal dysfunction. We also suggest that this may be the pathogenic basis for the significant incidence of late onset, isolated ureteral stenosis observed in these recipients. (*Am J Pathol* 1999, 154:1273-1284)

Polyoma viruses are almost ubiquitous and harmless in healthy humans but can cause clinically overt disease in immunocompromised individuals.¹ In transplant recipients and AIDS patients, BK polyoma virus has caused renal failure due to severe acute interstitial nephritis,²⁻⁷ distal ureteral stenosis,⁸⁻¹¹ hemorrhagic cystitis,¹²⁻¹⁵ desquamative interstitial pneumonitis,¹⁶ upper respiratory tract infections,¹⁷ and meningoencephalitis.^{16,18} JC polyoma virus also infects the kidney, but most commonly causes progressive multifocal leukoencephalopathy.¹⁹

Polyoma viruses, along with papilloma viruses, belong to a family of circularized, double stranded DNA viruses called Papovaviridae.²⁰ Most of these viruses have a narrow host range and do not productively infect other species. Several polyoma viruses infect old world primates (SV40, simian agent 12, polyoma virus papionis-2, and lymphotropic papovavirus). All members of this fam-

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ily have a similar genome organization with highly conserved regions and capsids that are of the same size and made up of three similar viral capsid proteins. The primate polyoma viruses share the use of two common regulatory proteins, which are transcribed early, known as the large T and the small T antigens.

SV40 virus, which is related to BK and JC viruses, has been reported in four cases of acute interstitial nephritis in rhesus monkeys (*Macaca mulatta*).^{21–23} Two with naturally acquired simian immunodeficiency virus (SIV) infection had mild SV40 infection in the kidney.²³ One experimentally infected with SIV developed severe acute tubulointerstitial nephritis 6 months later, with polyoma virus particles and DNA in renal tubular epithelial cells.²¹ A fourth animal, not known to be immunodeficient, developed interstitial pneumonia and “renal tubular necrosis” with viral particles and antigens in tubular cells.²² SV40 in oligodendrocytes was also noted in association with progressive multifocal leukoencephalopathy-like lesions in three other animals with SIV.²¹ Ureteral involvement was not described. SV40 virus contaminated early polio vaccines and its DNA sequences have been detected in sporadic human tumors; however, recent epidemiological data do not detect an association of SV40 and human tumors.²⁴

In the course of analyzing the renal allograft pathology in a cynomolgus monkey (*M. fascicularis*) treated with cyclosporine A and azathioprine, we noted enlarged atypical nuclei in renal cortical tubular epithelium that resembled BK virus infection in humans. Further investigation of the renal allograft revealed that the virus was a member of the polyoma virus group, as judged by the presence of positive immunoreactivity for the large T antigen of SV40, ultrastructure typical of polyoma virus and polymerase chain reaction (PCR) amplification of conserved polyoma sequences. Subsequently, similar observations were noted among monkeys treated on other protocols. We then proceeded to use the monoclonal antibody reactive to the large T antigen to ascertain the prevalence and pathological features of polyoma renal infection, with particular attention to the ureteral stenosis we had noted in some long term grafts in a tolerance protocol.²⁵

We report here, in cynomolgus monkeys treated with different immunosuppressive protocols for renal transplantation, the demonstration of a new species of polyoma virus infection that caused interstitial nephritis in autologous and transplanted kidneys, ureteritis and enteritis. These cases provide new insights into pathogenetic mechanisms that are potentially relevant to polyoma virus infections in humans.

Materials and Methods

Cynomolgus Recipients

Clinical details of the immunosuppressive protocols have been previously published. The immunosuppressive protocols included conventional immunosuppression (cyclosporin A (CsA), azathioprine), various monoclonal anti-

bodies (anti-CD3, anti-CD4, anti-CD54),^{26,27} and antagonists to adhesion molecules and cytokines. Mixed chimerism protocols to induce tolerance typically included pretransplant conditioning with whole-body and thymic irradiation and antithymocyte globulin (ATG); at the time of transplant donor bone marrow administration and splenectomy; and then one month of CsA.^{25,28} The cynomolgus monkeys were quarantined for 5 weeks before any immunosuppressive therapy and housed in other rooms in the same facility over a period of 2 to 8 months during treatment. The animals, from a colony in Mauritius, were purchased from Charles River Primate Center (Wilmington, MA) and received in groups that averaged 14 animals. The monkeys were maintained according to National Institutes of Health guidelines for the care and use of primates. One animal was housed per cage, with the cages stacked in groups of four, so that any animal might be able to have minimal contact with at most three other animals. All animals were transferred to adjacent procedure rooms as necessary for phlebotomy and other invasive procedures according to the particular treatment protocol. The monkeys did not encounter any other species during the protocol time period. Baboons from Charles River Primate Center were used as xenograft donors and were kept separated from the monkeys and other species. Animals are phlebotomized twice a week until renal function stabilizes and if clinically indicated undergo open renal biopsy. Autopsies were performed on all animals.

Selection of Cases

From January 1997, when the first case was detected, to October 1997, all tissue samples of native and transplanted kidneys and ureters (19 animals) were screened prospectively for polyoma virus by histology and polyoma virus antigen (see below). Other tissues studied for cytopathic change included liver, lung, and gastrointestinal tract. In addition, to determine whether cases had been overlooked previously, all renal biopsies and necropsy specimens from animals transplanted from 1991 to 1996 were screened retrospectively for evidence of polyoma cytopathic change (suspicious nuclear inclusions or enlargement) or atypical features of rejection (increased plasma cells in the infiltrate), which were found in 33 renal graft samples from 23 animals. Necropsies were performed typically within 12 hours of death or immediately after euthanasia. Additional samples with presumptive rejection (but without atypical features) were selected at random (20 samples from 14 animals). Overall, viral probes were tested on one or more samples from 50 allograft and 6 xenograft recipients, as well as 1 animal who received the immunosuppressive conditioning regimen for induction of mixed chimerism but without an organ transplant.

Histology and Immunocytochemistry

Tissue was fixed in 10% formalin, then dehydrated and embedded in paraffin per standard protocols. Sections

were stained with hematoxylin and eosin and with periodic acid-Schiff base (PAS). Viral antigens were detected in formalin-fixed paraffin embedded tissue, using the avidin-biotin-peroxidase complex technique.²⁹ Sections were mounted on Fisher Superfrost plus slides, deparaffinized in xylene, rehydrated in ethanol and phosphate-buffered saline, incubated for 5 minutes in 3% H₂O₂ in methanol to block endogenous peroxidase. Tissue sections were then heat-treated in 0.05 mol/L Tris-HCl, pH 10.0, for 10 minutes in a microwave oven, followed by incubation at room temperature for 20 minutes with avidin D (100 µg/ml) and biotin (10 µg/ml) to block endogenous biotin. Slides were stained overnight at 4°C with a monoclonal antibody to the large T antigen of SV40 (Ab-2, IgG2a; Oncogene Science, Calbiochem, Cambridge, MA), followed by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 35 minutes and then incubated in preformed avidin-biotinylated horseradish peroxidase complexes (Elite ABC, Vector Laboratories). Tissue sections were washed and developed with 3-amino-9-ethyl-carbazole (Aldrich, Milwaukee, WI), counterstained with Gill's hematoxylin and mounted in glycerol (DAKO, Carpinteria, CA). An IgG2a isotype control was used, which showed no specific nuclear staining (anti-CD3, NCL-CD3-PS1, Novacastra). Overall, 113 specimens from 57 animals were stained for virus, including samples of native kidney (17), allograft kidney (67), allograft ureter (11), xenograft kidney (10), xenograft ureter (2), liver (2), intestine (3), and lung (1). Cases with cytopathic changes suspicious for cytomegalovirus were stained by a similar technique using monoclonal antibodies to immediate early antigen (Chemicon, Temecula, CA) and delayed early antigen (DAKO).

Electron Microscopy

Tissue from the first case (M2396) was retrieved from paraffin, dewaxed with xylene, rehydrated, and fixed in Karnovsky's fixative (K2) for 1.5 hours, prestained with uranyl acetate, embedded in Epon resin, sectioned, and counterstained with lead citrate.³⁰ Sections were examined in a Philips 301 electron microscope.

Polymerase Chain Reaction

DNA was extracted from tissue saved frozen at -80°C in OCT (Fisher Scientific). Frozen tissue samples (approximately 0.5 to 1 g) from an allograft (M1996), a xenograft (M3996), a native kidney (M3996), and a normal cynomolgus kidney were ground with a mortar and pestle in liquid nitrogen. The ground tissue was transferred to 2.5 ml of 75 mmol/L NaCl and 25 mmol/L EDTA, pH 8.0; 2.5 ml of 10 mmol/L Tris-HCl, 100 mmol/L NaCl, 10 mmol/L EDTA, pH 7.5, was added with 0.3 ml of 20% SDS and 0.2 ml of 10 mg/ml of proteinase K and allowed to digest at 55°C overnight. The DNA was extracted three times with saturated phenol, three times with 20:1 chloroform-isoamyl alcohol and precipitated with 100% ethanol at -20°C. The DNA was pelleted at 15,000 rpm for 30 minutes and resuspended in 1 ml of TE buffer, pH 8.0.

Primers for the SV40 large T antigen were slightly modified to account for minor differences in homology in the same region for BK and JC viruses, previously described.³¹ For polymerase chain reaction (PCR) amplification, 1 mg of total cellular DNA was incubated with *Thermus aquaticus* DNA polymerase (Amplitaq, Perkin Elmer, Norwalk, CT) with 1.5 mmol/L Mg²⁺ and primer concentrations of 1 mmol/L under the following conditions: 92°C for 3 minutes, 92°C for 30 seconds, annealing at 52°C for 30 seconds, elongation at 72°C for 30 seconds for 35 cycles. Primer sequences (56 to 36) are as follows: PYV.for, TAG GTG CCA ACC TAT GGA AC; PYV.rev, GGA AAG TCT TTA GGG TCT TCT ACC.³¹ Amplified products were run on 2% agarose gels and stained with ethidium bromide. The portion amplified represents 129 bases numbered 4425-4554 in the SV40 sequence as listed in the EMBL gene database (www.srs.ebi.ac.uk). Purified amplified DNA was sequenced using the Big Dye Terminator Fluorescence Sequencing technique (Perkin Elmer) and an ABI377 Automatic Fluorescence Sequencer (Perkin Elmer).

Results

Twelve cynomolgus monkeys had polyoma virus interstitial nephritis in the native kidney and/or the renal graft (Table 1). Polyoma virus infected six allografts and one xenograft, as well as six native kidneys.

Native Kidneys

Polyoma virus was detected in five native kidneys of 16 tested, on days 11 to 33 after renal transplantation and native ureteral ligation (Table 2). In three of these recipients, the transplant kidney was not infected; the one graft infected simultaneously was a baboon xenograft. One additional animal (M297) with polyoma virus infection of the native kidney had not received a renal transplant but had the mixed chimerism conditioning regimen 52 days previously (Figure 1, A and B). This native kidney was particularly instructive, since the ureter was not tied. The kidney had nuclear changes in collecting ducts associated with a patchy infiltrate rich in plasma cells (Figure 1, A and B). This animal had a mild renal dysfunction (serum creatinine of 1.3 mg/dl *versus* the normal monkey level of 0.5 to 0.8 mg/dl).

The most consistent morphological sign of the virus was the enlargement (two- to threefold) of tubular cell nuclei, with a hyperchromatic appearance, sometimes with inclusions that resembled enlarged nucleoli or were homogeneously glassy and lavender on H&E (Figures 1C and 2A). Many more nuclei contained polyoma virus large T antigen than had light microscopic viral inclusions; the number of positive cells ranged from about 4-60 per 10x power field (2.4 mm²) (Figure 1D). Viral antigen was present in a fine particulate or dense homogeneous pattern in the nucleus; no cytoplasmic staining was found (Figure 2B). Viral antigen was detected primarily in collecting duct epithelium in the cortex and medulla; overall, about 1 to 5% of the tubular cross sections were antigen positive (Figure 1D). The most ad-

Table 1. Cynomolgus Monkeys with Polyomavirus Infection

Recipient	Therapy/protocol*	Clinical presentation	Site	Day	Large T antigen	Group No.
Allografts						
M2396	Cyclosporin/azathioprine	Renal dysfunction	Allograft kidney	79	+	2-96
M3993	Mixed chimerism	Renal dysfunction	Allograft kidney	37	+	n.a.
M396	Mixed chimerism	Renal dysfunction	Allograft kidney	29	+	1-96
			Allograft kidney	35	+	1-96
			Allograft ureter	35	+	1-96
M1996	Mixed chimerism	Renal dysfunction	Allograft kidney	21	++ [†]	2-96
			Allograft ureter	21	+	2-96
			Native kidney	21	-	2-96
			Colon	21	-	2-96
M1097	Mixed chimerism	Renal dysfunction	Allograft ureter	20	+	1-97
			Allograft kidney	20	-	1-97
			Native kidney	23	+	1-97
M3096	Mixed chimerism	Lung abscess	Allograft kidney	29	+	2-96
			Allograft kidney	34	+	2-96
			Native kidney	34	-	3-96
			Colon	34	-	3-96
M997	Mixed chimerism	Postoperative acute tubular necrosis	Allograft kidney	36	+	1-97
M1397	Mixed chimerism	Renal dysfunction	Allograft kidney	90	-	1-97
		Sepsis	Allograft kidney	9	-	3-96
			Allograft kidney	11	-	3-96
			Native kidney	11	+	1-97
Xenografts						
M3996	Mixed chimerism	Renal dysfunction	Native kidney	31	++ (CMV+)	3-96
		Diarrhea, skin rash	Xenograft kidney	31	++ (CMV+)	Bab
		(?) graft-versus-host disease	Small Intestine	31	+	3-96
			Colon	31	+	3-96
			Liver	31	-	3-96
M2696	Mixed chimerism	Renal dysfunction	Native kidney	33	+	3-96
			Xenograft kidney	33	- (CMV+)	Bab
			Xenograft ureter	33	- (CMV+)	Bab
M3296	Mixed chimerism	Renal dysfunction	Native kidney	0	-	3-96
			Native kidney	22	+	3-96
			Xenograft kidney	22	-	Bab
			Xenograft ureter	22	-	Bab
No graft						
M297	Mixed chimerism	Pancytopenia	Native kidney	52	+	1-97
			Liver	52	-	1-97
			Lung	52	-	1-97

*Described under Methods.

[†]Confirmed by PCR.

CMV, cytomegalovirus.

vanced cases had virus in all other tubular segments, as well as Bowman's capsule. The infected cells tended to be in contiguous clusters within individual tubular cross-sections; up to 100% of the nuclei (eg, 24/24 nuclei of a single tubular cross section) were positive. The interstitial infiltrate of lymphocytes and plasma cells was most evident around involved infected tubules, which also showed tubulitis and apoptosis. Plasma cells invaded tubules, a feature not described in other forms of interstitial nephritis (Figure 1B). Detachment from the substrate basement membrane (anoikis) was prominent; the detached cells usually contained viral antigens (Figure 2B). Focal destruction of tubules was sometimes prominent, with dissolution of the tubular basement membrane on PAS stain, which also revealed deposits of PAS⁺ cast material (Figure 2C). The elongated shape, large diameter and location suggested that the destroyed tubules

had been collecting ducts. Endothelialitis or glomerular lesions were not observed.

None of the native kidneys had detectable viral antigens at the time of transplantation or after day 52

Table 2. Timing of Polyomavirus Infection after Transplant

Day post-transplant	Allograft		Native kidney	Xenograft	
	Kidney	Ureter		Kidney	Ureter
0	0/2		0/4		
1-20	0/12	1/2	2/3		
21-48	8/22	2/3	3/7	1/3	0/1
49-72	0/15		1/1	0/3	
73-100	1/6	0/3	0/2	0/4	0/1
101-824	0/9	0/3	0/1		

Results are number of polyoma large T antigen positive samples/number tested.

Figure 1. Light microscopy of polyomavirus infection in autologous kidneys. A widespread, focally intense mononuclear and plasma cell infiltrate surrounds infected tubules with characteristic nuclear changes (arrows) is seen (A), which at higher power (B) reveals an occasional plasma cell inside tubules (arrow) and epithelial cell nuclear changes are evident (arrowheads). C: Characteristic nuclear changes are seen many collecting duct epithelial cells, including several that have become detached (anoikis). D: A low power view of the medulla shows the extensive nuclear polyoma antigen by immunocytochemistry using a monoclonal antibody to SV40 large T antigen (see under Methods). A and B: A non-transplanted monkey, M297. C and D: The native kidney of M3996.

(Table 2). One was negative on day 0 and positive on day 22. All kidneys with ligated ureters also had features of chronic obstruction (interstitial fibrosis, tubular dilation and atrophy and a mild mononuclear infiltrate). When the pathology of the negative native kidneys was compared with those that had polyoma infections, the differences noted were more infiltrate and tubular destruction in the infected kidneys.

Allografts

Six renal allografts had polyoma virus infection, in 8 samples taken 21 to 79 days after transplantation (mean 36 ± 17 days) (21,29,29,33,34,36,37,79 days). The peak frequency of infection was from day 21–48 after transplant (Table 2). None of the allografts had detectable virus before 21 days. As in the native kidneys, the virus was detected in collecting tubule epithelium in the cortex and medulla (Figure 2, A and B). The nuclear changes were sometimes subtle. In contrast to the native kidneys, some of the grafts had endarteritis, indicative of active cellular rejection (M396, M1996, M1097, and M3096). The interstitial infiltrate was more intense and focal hemorrhage was noted, also suggesting a component of rejection (Figure 2A). Tubular cell apoptosis was more conspicuous than is typical of graft rejection. Plasma cells were

found in tubules, a feature not described in graft rejection. Several grafts had extensive rupture and destruction of collecting ducts, which is also uncommon in graft rejection (Figure 2C) (M396, M2396, M3096). Tubular calcifications sometimes contained nuclear remnants that stained for virus. None of the animals with detectable virus in the allograft had infection of the native kidney. One graft (M997) was positive on day 36 and negative on day 90.

The donor ureter had detectable polyoma virus in three cases (days 20, 21 and 35) (Figure 3). Viral antigen was detected in scattered urothelial cells. In two cases viral antigen was in the nuclei of smooth muscle cells of the muscularis propria (days 21, 35), associated with apoptosis of smooth muscle cells and acute ureteritis with mononuclear inflammation. Two of the three animals had viral antigen in the allograft kidney and the other had virus in the native kidney. These animals had presented with rising creatinine and had died on post-transplant days 19–37. All three had acute cellular rejection (with endarteritis) in both the ureter and kidney (M396, M1996 and M3096). Virus was not detectable in eight other transplanted ureters, sampled from 7 to 173 days (81 ± 64 days) after transplantation (Table 2). Five of these had chronic ureteritis of varying severity with fibrosis. One had endarteritis; two were unremarkable.

Figure 2. Light microscopy of polyoma virus infection in allograft kidneys. The nuclear and destructive changes are similar to those in the native kidneys, although often the infiltrate is more pronounced. **A:** Prominent plasmocytic and mononuclear peritubular infiltrate with hemorrhage surrounds a tubule with basophilic viral nuclear changes and apoptosis. **B:** Immunocytochemistry demonstrates polyoma large T antigen in nuclei of collecting duct epithelial cells in a fine particulate or dense homogeneous pattern. No cytoplasmic staining is seen. Note the faintly staining cells in the lumen of a tubule (arrowhead). **C:** Rupture and destruction of collecting ducts, not usually seen in graft rejection, is evident (arrowhead). **A and B:** M1996. **C:** M396.

Xenografts

One baboon xenograft kidney studied on day 31 had focal polyoma infection in a pattern similar to that observed in the allografts (Figure 4). The native kidney in the xenograft recipient revealed a much more widespread infection than that observed in the transplant. Of the nine

samples from five other baboon renal xenografts studied from day 22 to 88, none had detectable polyoma virus infection; two had infection of the native cynomolgus kidney. None of the two xenograft ureters had detectable SV40 virus (days 33 and 86).

Other Organs

Polyoma virus antigen was prominent in the muscularis propria of the small and large intestine in a xenograft recipient with severe diarrhea which had been attributed to possible graft-versus-host disease (M3996) (Figure 5). Inflammation and apoptosis in the smooth muscle were present in association with the infected cells. Virus was not detected in the epithelium of the gastrointestinal tract, in the liver or lung. Sections of colon were negative on two other animals which had viral antigen in the kidney at the time of death; samples from liver and lung were negative in other animals (Table 1).

Electron Microscopy

Virions with a morphology compatible with polyoma virus were detected in the nuclei of tubular cells (Figure 6). The virions were closely packed, non-enveloped dense spherical particles 30 to 32 nm in diameter. Cytoplasmic particles were not seen. The usual diameter described for SV40 is 40 to 45 nm,^{21,23} although a human case of BK virus had 32 nm virions.⁵ Probably variations in specimen preparation, including retrieval from paraffin, affect the measured size.

Polymerase Chain Reaction

Polyoma virus DNA was detected by PCR in cases in which viral antigens were detected by immunohistochemistry using primers from a highly conserved region of the large T antigen (Table 1) (Figure 7). The amplified product was similar in size to that of SV40 and the human polyoma viruses, BK virus, and JC virus. No polyoma virus DNA was detected in a control cynomolgus monkey kidney DNA after 35 cycles of amplification.

Partial Sequence of the Large T Antigen Gene

The sequence of the amplified portion of the large T antigen from three cases studied showed 90 to 92% bp homology with the SV40 reference sequence. We found 7/126 nucleotide substitutions as well as four deletions that were identical in each isolate. In addition two of three isolates had an additional base deletion. Two of the observed deletions were also found in the SV40 strain amplified (K661) compared with the GenBank sequence. Overall, the kidney isolates had a calculated homology at the amino acid level of 84% in this portion of the large T antigen compared with SV40 (6 substitutions had no coding change). The corresponding homology with JC virus was 68% and with BK virus 62% (JC is 80% homologous to BK in this region) (Figure 8B).

Figure 3. Polyoma ureteritis in an allograft (M396). **A:** A mixed lymphocytic and plasmocytic infiltrate extends throughout the muscularis and focally into the mucosa (top). Nuclear changes are difficult to appreciate at this magnification. **B:** Immunocytochemistry show polyoma large T antigen in nuclei of scattered cells in the urothelium (arrows) and in the muscularis propria (arrowhead). At higher magnification apoptotic smooth muscle cells and cells with characteristic nuclear changes are evident (**C**); many of the smooth muscle cell nuclei in these areas have stain for polyoma large T antigen (**D**).

Retrospective Screen

To determine whether the viral infection had been overlooked in previous biopsy material with acute cellular rejection, 33 archival graft samples from 23 animals with increased plasma cells in the infiltrate or suspicious tubular epithelial nuclear changes were stained for polyoma virus. A single positive case was identified which had one cluster of positive tubular nuclei (animal M3993, Table 1); even in retrospect, no viral inclusions or nuclear changes were detected on routine H&E stains. A similar screen of 20 random samples from 14 additional animals from 1992–1996 detected no polyoma virus antigens. Overall the increased yield with antibody was 1.9%.

Figure 4. Light microscopy of polyoma virus infection in a xenograft kidney (M3996). **A:** The nuclear changes and destruction of tubules are similar to those seen in allografts, although less severe than in the native kidney of the same animal. The extent of the viral infection, as indicated by large T antigen (**B**), is also less than in the native kidney (compare Figures 1C and 1D).

Figure 5. Polyoma infection of the small intestine muscularis propria (M3996). **A:** Scattered enlarged smooth muscle cells with enlarged, glassy, basophilic nuclei (arrows) are reminiscent of the nuclei of CPV-infected renal epithelial cells. Focal apoptosis is evident (arrowheads). The smooth muscle nuclei contain polyoma large T antigen (**B**).

Figure 6. Electron micrographs of CPV infected tubular epithelial cells (M2396). **A:** Early apoptotic nuclear changes include irregular clumping of chromatin around the nuclear envelope; collections of moderately electron dense viral particles are in the nucleus (arrowheads); $\times 6100$. Higher magnification reveals paracrystalline arrays of round 30–32 nm virions consistent with polyoma virus (**B**). $\times 34,200$; scale bar, 200 nm.

Clinical Features

Most animals that had polyoma virus infection histologically had rising creatinine as an indicator of infection; this clinical picture was indistinguishable from acute cellular rejection and was often accompanied by lethargy and anorexia. Other manifestations at the time of biopsy or death included pancytopenia, lung abscess, and symptoms suggesting graft-versus-host disease (macular skin eruption and diarrhea). Two animals were co-infected with cytomegalovirus (M3996, M2696) and one (M997) had lymphoproliferative disease in lymphnodes that expressed Epstein-Barr virus related products (submitted for publication).

No particular association of polyoma virus with any of the immunosuppressive protocols was evident, although the numbers are small (Table 3). However, the infection appears to be more prevalent since 1996: 5 of 6 of the infected grafts were from 1996–1997, as well 3/3 of the

Figure 7. 2.0% agarose gels of PCR amplification products. **Lanes 1–6** were amplified using control primers for a macaque immunoglobulin gene (see under Methods). **Lanes 7–14** were amplified using the PYV.for and PYV.rev modified large T antigen primers. Approximately 1 μ g of SV40 DNA (SV), BK and JC viral DNA were amplified; note that the amplification products for BK and JC are slightly larger than for SV40. 1 mg of total cellular DNA extracted from fresh frozen kidney tissue was amplified. P2 is an allograft kidney (M1996); P4 is the native kidney and P5 is the baboon xenograft from M3996; P6 is kidney tissue from an untreated control monkey. **Lanes 1 and 7** represent negative controls. The marker lane is a *Hae*III digest of pUC plasmid DNA.

positive ureters and 5/5 of the infected native kidneys (Table 4). The positive cases in 1996–1997 were received in 4 of 7 batches (deliveries) of animals. Other animals received in those batches and which underwent identical or similar immunosuppressive regimens did not show signs of infection or did not stain for the large T antigen.

Discussion

These studies identify a new polyoma infection in cynomolgus monkeys and provide insights into analogous infections in humans. The electron microscopy, antibody, and genomic probes for the large T antigen identify the monkey virus as a member of the polyoma virus family. The large T antigen is highly conserved among SV40, BK, and JC viruses with up to 75% identity, likely representing a common ancestral origin.³² The large T DNA sequences amplified from the monkey kidneys show 90 to 92% genomic and 84% amino acid homology to the corresponding portion of the SV40 genome and lesser homology to BK and JC virus. The amino acid homology between JC and BK in this region is similar (80%) to that between SV40 and the present isolates. We have concluded therefore that this virus is a new member of the polyoma virus family, closely related to SV40, and have termed it the cynomolgus polyoma virus (CPV). Supporting this conclusion is the observation that substantial other genetic differences exist, including a large deletion (M. A. van Gordier, J.W. Henson, and R.B. Colvin, unpublished observations).

Four histopathological phases were recognized, the initial phase (I) showing prominent viral antigens in collecting duct nuclei, and a mild associated interstitial lym-

A

Cynomolgus Polyoma Virus Large T Antigen PCR Sequences Compared with SV40

Source	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
SV40 Standard (EMBL)	T	GAT	GAA	TGG	GAG	CAG	TGG	TGG	AAT	GCC	TTT	AAT	GAG	GAA	AAC	CTG	TTT	TGC	TCA	GAA	GAA	ATG
Amino Acid Sequence	Asp	Glu	Trp	Glu	Gln	Trp	Trp	Asn	Ala	Phe	Asn	Glu	Glu	Asn	Leu	Phe	Cys	Ser	Glu	Glu	Met	
SV40 PCR Control (K661)	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Allograft Kidney (M1996)	---	---	---	---	---	---	---	---	---	---	---	---	---	*	**	---	---	--T	---	---	---	
														Asp								
Native Kidney (M3996)	---	---	---	---	---	---	---	---	---	---	---	---	---	*	**	---	---	--T	---	---	---	
Xenograft Kidney (M3996)	---	---	---	---	---	---	---	---	---	---	---	---	---	*	**	---	---	--T	---	---	---	
SV40 Standard (EMBL)	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
CCA	TCT	AGT	GAT	GAT	GAG	GCT	ACT	GCT	GAC	TCT	CAA	CAT	TCT	ACT	CCT	CCA	AAA	AAG	AAG	AGA	AA	
Amino Acid Sequence	Pro	Ser	Ser	Asp	Asp	Glu	Ala	Ser	Ala	Asp	Ser	Gln	His	Ser	Thr	Pro	Pro	Lys	Lys	Lys	Arg	
SV40 PCR Control (K661)	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	*	---	*	---	---
Allograft Kidney (M1996)	---	---	C--		--A		---	---	---	---	---	---	---	--A	--C	--C	--T	*	---	Arg	Glu	Lys
			Arg		Glu															Arg	Glu	Lys
Native Kidney (M3996)	---	---	C--		--A		---	---	---	---	---	---	---	--A	--C	--C	--T	*	---	Arg	Arg	Glu
Xenograft Kidney (M3996)	---	---	C--		--A		---	---	---	---	---	---	---	--A	--C	--C	--T	*	---	Arg	Glu	Lys

B

Comparison of CPV large T antigen amino acid sequences with other polyomaviruses

SV40:	DEWEQ	WNNA-	NEEN*	**LFC	SEEMP	SSDDE	ATADS	QHSTP	PKKKR
CPV:	-----	-----	--*D	**---	-----	-R-E-	-----	-----	--REK
JCV:	---S	---T-	--KWD	ED---	H---F	A----	N-*G-	-----	---K
BKV:	E---S	--SS-	--KWD	ED---	H-D-F	A--E-	-----	-----	-----

Figure 8. (A) Sequences of the PCR amplified products from the large T antigen genome from three isolates compared with GenBank SV40 sequences and those amplified from a laboratory reference strain (K661). The 5'-3' coding strands are shown with the corresponding amino acids for the sequences between the primers. Identical nucleotides are indicated by -, deletions by *, according to optimized alignment. Overall there is 90 to 92% nucleotide and 83% amino acid homology. (B) Amino acid sequences of the portion of CPV amplified compared with corresponding portions of SV40, BK and JC viruses given in standard single letter abbreviations. The CPV is 84% homologous with SV40, 62% with BK and 68% with JC viruses respectively in this region (JC and BK are 80% homologous with each other).

phocytic and plasmocytic infiltration. The cytolytic phase (II) is characterized by apoptosis, anokis, and tubulitis in collecting ducts, later extending to other tubules. A destructive phase (III), manifested by dissolution of collecting ducts (loss of integrity of the tubular basement membrane, loss of normal collecting duct epithelium), is found in those more severe infections in which renal failure develops. A late phase (IV) has tubular scarring and calcification, sometimes with viral antigen in foci of destroyed tubules. This may be hard to recognize as related to polyoma virus, even with immunohistochemistry.

The pathology of the viral infection has striking similarity to BK virus infections in humans.²⁻⁴ In humans the nuclei of infected tubular cells are usually enlarged and

tend to be grouped in tubules, particularly collecting ducts in the cortex and outer medulla, and can often be spotted at low power. The mononuclear interstitial infiltrate, associated with infected cells, often contains plasma cells. In both the human and monkey studies the plasma cells sometimes invade the tubules, a feature not seen in allograft rejection or other forms of interstitial nephritis.³³ The pathological findings are also compatible with the four previously published cases of SV40 induced interstitial nephritis in rhesus monkeys.²¹⁻²³

In our tolerance protocols, distal ureteral stenosis sometimes complicated the course, typically 2 to 4 months after transplantation.^{25,28} The pathogenetic mechanisms considered included rejection, ischemia, and polyoma virus, none of which seemed certain. Since several allografts lacked evidence of rejection in the kidney, it was difficult to conclude that chronic rejection restricted to the ureter was operative. An ischemic origin also seemed unlikely, since the onset was delayed by 2 months or more after transplantation. Finally, no virus was detectable immunochemically in those ureters with late stenosis. Ureteral stenosis in humans has been attributed to the BK virus, which was originally isolated from a patient with distal donor ureteral stenosis, 3 months after transplant.⁸ At least five subsequent patients have been reported with ureteral stenosis.⁹⁻¹¹ The BK virus was

Table 3. Frequency of Allograft Polyomavirus Infection by Treatment

Treatment protocol*	Negative†	Positive†	%‡
Cyclosporine/azathioprine	6 (6)	1 (1)	17
Mixed chimerism	28 (43)	6 (8)	18
Monoclonal antibodies	4 (4)	0	0
Other	4 (9)	0	0

*Described under Methods.

†N recipients (N samples).

‡Percentage of animals with polyoma large T antigen positive in one or more samples.

Table 4. Chronology of Polyomavirus Infection

Year of transplant	Allograft				Native		Xenograft			
	Kidney		Ureter		kidney		Kidney		Ureter	
	+	–	+	–	+	–	+	–	+	–
1991–1992	0	3			0	1				
1993	1	13	0	1						
1994	0	3	0	1						
1995	0	9	0	4	0	1	0	1		
1996	1	5	1	0	0	1	0	1	0	1
1997	4	8	2	2	6	8	1	3	0	1
Subtotals										
1991–1995	1	30	0	5	0	2	0	1		
1996–1997	5*	12	3	3	5	9	1	4	0	2

Results are number of animals by year of transplant.

* $p = 0.0166$ 1996–1997 vs 1991–1995 for allograft kidneys (Fisher exact test).

demonstrated in the ureteric epithelium, which was often sloughed; some cases had destruction of the ureteric wall with replacement with granulation tissue.⁹

Here we identified ureteral viral infection in three subjects studied in the first 2 to 7 weeks after transplant (in one case before the kidney was infected) and before ureteral stenosis typically appears. A novel finding was the documentation of viral infection and injury of the ureteral smooth muscle, which showed marked apoptosis. The destruction of the muscular wall with attendant inflammation noted here may be relevant to the later development of stenosis. Thus, in the ureter three pathophysiological phases are postulated. The initial phase involves focal infection of the urothelium, which may be incidental. The destructive phase includes widespread infection of urothelium and smooth muscle, with ulceration and marked inflammation, which is probably the prelude to ureteral stenosis. Rejection probably also contributes to this injury; one of the best arguments for this is that no cases of ureteral stenosis have been reported in non-renal transplant recipients with polyoma cystitis. We propose that ureteral stenosis is the late, third phase of the process at which point the virus may no longer be demonstrable, as found in our studies. To our knowledge ureteral polyoma virus infection has not been previously described in non-human primates.

It is difficult to determine in biopsies from allografts with impaired function and virus infection whether the principal process is infection or rejection, since both are characterized by mononuclear inflammation and tubulitis. The most discriminating features in our opinion are 1) widespread viral cytopathic changes/antigen; 2) collecting duct destruction, which is more typical of polyoma infection; and 3) endarteritis, which is pathognomonic of active and more severe rejection. We found that inflammation characteristically affects the medulla and polyoma virus should be considered whenever that is prominent. In our animals we attributed graft loss to polyoma virus in two (M396, M1996) and death in one animal (M3996). These had numerous viral infected cells, intense inflammation, extensive collecting duct destruction, and marked ureteral infection. These also had focal endarteritis (M396, M1996), and thus had an element of rejection. One animal recovered (M997), as judged by

lack of viral antigens, without specific therapy (the animal had been off all immunosuppressant drugs for 2 months at the time of the second sample). Notably, no CPV infection persisted beyond 3 months after transplantation, suggesting recovery of resistance.

The clinical features of CPV infection are similar to BK virus infection in humans. The time course resembles that in patients undergoing bone marrow transplant.¹⁴ While renal polyoma virus infections are usually self-limited and mild, a few cases have progressed to renal failure.^{5,6} In one renal transplant recipient, the disease presented as refractory interstitial nephritis that resulted in an allograft nephrectomy; another, interpreted as acute cellular rejection, responded to immunosuppression, but recurred twice on subsequent follow-up.³ Reduced immunosuppression has led to recovery in several cases of BK virus in humans. Two cases had transient azotemia that resolved with conversion from FK506 to CsA.² In a prospective study of renal transplant recipients, active polyoma virus (BK or JC) infection was shown in 65%; renal function became impaired at the time of the infection in about 25%, but no biopsy was done.¹⁰

Some of our findings have not been previously observed in humans. For example, we found that the native kidney may be the principal site of infection in transplant recipients, a feature not previously appreciated. It is known that native kidneys can be infected in primary immunodeficiencies. The native kidney should be considered a potential site in human transplant recipients with an unexplained fever or a suspected viral infection. Second, the gastrointestinal tract was shown to be infected by polyoma virus. We found that the virus could infect smooth muscle cells of the gastrointestinal tract with associated apoptosis and inflammation. The clinical syndrome may be confused with graft-versus-host disease or a bacterial enteritis. Infection of vascular smooth muscle, as judged by immunohistochemistry, has been illustrated in a BK virus infection of the leptomeninges.¹⁶ *In situ* hybridization would be useful to confirm these findings.

In human bone marrow recipients, polyomaviruria occurs exclusively in patients who are seropositive at the time of transplantation.¹⁴ The epidemiology in these monkeys also suggests reactivation, although we have no

serological data. All renal infections occurred between 3 weeks and 3 months after transplantation. This is a time of increased risk for several latent viral infections in humans (including polyoma virus). Also supporting reactivation disease is the lack of concordance between the native and allograft infection, since both kidneys should be susceptible to exogenous infection. Some of the native kidneys were infected without involvement of the allograft, suggesting the recipient harbored the latent virus. Conversely, those cases in which only the allograft was infected may have been carried by the allograft itself, as in humans, in whom a seropositive donor increases the rate of primary or reactivation infections with BK virus.³⁴ In the case of the baboon xenograft which was infected simultaneously, but less severely than the native cynomolgus kidney, the molecular data support a common origin, probably transmitted from host to graft.

A puzzling epidemiological aspect was the increased prevalence in the last two years. Our screening of archival cases revealed a single previous case (<2%). The immunosuppressive protocols we have tested over this period of time have varied, but the most common current protocol (mixed chimerism) has been used since 1992. However, the frequency of polyoma virus does not seem to correlate with the type of immunosuppression, which included conventional CsA and azathioprine and whole-body irradiation/ATG, and CsA. In humans BK virus infection has also occurred in patients receiving a variety of immunosuppressants from azathioprine to CsA and most recently tacrolimus.^{2,3} It is likely that susceptibility is not related to a particular treatment, but rather the overall degree of immunosuppression. It is possible that the prevalence of latent infection has increased in our subjects. All but one of the infected monkeys came from four shipments of animals received in 1995–1997. Further studies will be needed to determine whether the recent groups have a higher prevalence of latent infection.

The present study demonstrates that the collecting duct is a favored site of infection. Infection of collecting ducts of the medulla has also been observed in prior SV40 monkey studies, but with little comment.^{21,23} The human pathology literature is also relatively silent on the tubular site of infection. One report noted virus in all tubules in end stage BK infection.³ In another case, JC virus replication was demonstrated predominately in collecting ducts by *in situ* hybridization.¹⁹ Based on the cases one of us has reviewed, the collecting duct may also be the favored site, at least in mild or early infections.³³ The presumption is that those cells either have more abundant viral receptors or are more conducive to viral replication. It is perhaps relevant that the collecting ducts and the urothelium both originate embryologically from the metanephric duct.

The pathogenetic determinants of virulence probably lie both in the host and in the virus. In the host two factors are important: the state of the immune system and the state of the cells in the target organ. Infection of immunocompetent rhesus monkeys with SV40 results in viremia, viruria, and seroconversion without clinical symptoms.³⁵ Similarly, BK and JC virus rarely if ever cause overt disease in immunologically intact humans.¹ In mice

renal injury from toxins or ischemia enhances polyoma virus replication in the kidney³⁶ and analogous mechanisms may promote SV40 replication in obstructed or rejecting kidneys. Surface MHC class I molecules are required for SV40 polyoma virus infection *in vitro* and may participate in the internalization of the virus.³⁷ Up-regulation of these molecules due to cytokines during rejection or inflammation may promote transmission of the virus. Overt injury to the kidney is not necessary to trigger the viral infections in immunosuppressed subjects, as shown in the animal who had not received a transplant.

The causal relationship between rejection and viral infection is potentially bi-directional. It is well known in humans that viral infections, such as cytomegalovirus, promote acute rejection, probably in part by stimulating increased cytokine production.³³ A mechanism was demonstrated in recent studies of peripheral tolerance induction in transgenic mice, in which viral infection or exogenous cytokine administration blocks peripheral deletion and inactivation of self-reactive CD8⁺ cells and leads to graft-versus-host disease-like reactions.³⁸ Thus, viral infections, such as polyoma virus, may interfere with the success of clinical protocols for tolerance induction.

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