

## TESTING FOR POLYOMAVIRUS TYPE BK DNA IN PLASMA TO IDENTIFY RENAL-ALLOGRAFT RECIPIENTS WITH VIRAL NEPHROPATHY

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**ABSTRACT**

**Background** Reactivation of polyomavirus type BK (BK virus) is increasingly recognized as a cause of severe renal-allograft dysfunction. Currently, patients at risk for nephropathy due to infection with the BK virus are identified by the presence of cells containing viral inclusion bodies ("decoy cells") in the urine or by biopsy of allograft tissue.

**Methods** In a retrospective analysis, we performed polymerase-chain-reaction assays for BK virus DNA in plasma samples from 9 renal-allograft recipients with BK virus nephropathy; 41 renal-allograft recipients who did not have signs of nephropathy, 16 of whom had decoy cells in the urine; and as immunocompromised controls, 17 patients who had human immunodeficiency virus type 1 (HIV-1) infection (stage C3 according to the classification of the Centers for Disease Control and Prevention) and who had not undergone transplantation.

**Results** In all nine patients with BK virus nephropathy, BK virus DNA was detected in the plasma at the time of the initial histologic diagnosis (a mean [ $\pm$ SD] of  $46 \pm 28$  weeks after transplantation) and during the course of histologically diagnosed, persistent BK virus disease. In three of the six patients with nephropathy who were studied serially after transplantation, BK virus DNA was initially undetectable but was detected 16 to 33 weeks before nephropathy became clinically evident and was confirmed by biopsy. Tests for BK virus DNA in plasma became negative and the nephropathy resolved after the doses of immunosuppressive drugs were decreased in two patients and after removal of the renal allograft in three patients. BK virus DNA was found in the plasma of only 2 of the 41 renal-allograft recipients who had no signs of nephropathy and in none of the patients with HIV-1 infection.

**Conclusions** Testing for BK virus DNA in plasma from renal-allograft recipients with use of the polymerase chain reaction is a sensitive and specific method for identifying viral nephropathy. (N Engl J Med 2000;342:1309-15.)

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**T**HE human polyomaviruses BK virus and JC virus are nonenveloped viruses that have a diameter of 45 nm and a circular, double-stranded-DNA genome of 5300 bp.<sup>1</sup> Each of the two viruses is 70 percent homologous with the other and with simian virus 40 (SV40).<sup>1</sup> Typically, primary infection with the BK virus or JC virus occurs during childhood and does not produce spe-

cific symptoms; the prevalence on serologic testing is more than 80 percent among adults worldwide.<sup>1,2</sup> Serologic studies suggest that the BK virus and JC virus are transmitted independently of one another, most likely by the oral or respiratory route.<sup>1</sup>

After primary infection, the polyomaviruses remain latent in the urogenital tract.<sup>3</sup> Asymptomatic reactivation and intermittent shedding of virus in the urine occur spontaneously in immunocompetent persons but are more frequent among those with altered cellular immunity, such as pregnant women,<sup>3,4</sup> patients with cancer who are receiving chemotherapy,<sup>5</sup> patients with human immunodeficiency virus type 1 (HIV-1) infection,<sup>6,7</sup> and recipients of renal or other allografts.<sup>8,9</sup> Overt clinical disease from polyomavirus infection is rare and is clearly linked to the degree of immunocompromise.

One of the most debilitating diseases associated with this infection is progressive multifocal leukoencephalopathy, which is caused by the JC virus<sup>10</sup>; the incidence of this disease has increased since the beginning of the acquired immunodeficiency syndrome epidemic.<sup>11</sup> In recipients of bone marrow transplants, infection with the BK virus may cause hemorrhagic cystitis.<sup>9,12,13</sup> In recipients of renal allografts, increased replication of the virus is readily detectable in the urine by the presence of "decoy cells," which contain characteristic intranuclear viral inclusion bodies.<sup>14,15</sup> In four studies involving more than 600 renal-allograft recipients, reactivation of the BK virus was associated only inconsistently with ureteral stenosis or varying degrees of graft dysfunction.<sup>8,15-17</sup>

More recently, persistent polyomavirus replication in patients with renal allografts was identified as an important cause of progressive graft dysfunction and graft loss among those treated with immunosuppressive regimens that included tacrolimus or mycophenolate mofetil.<sup>18-22</sup> In these patients, histologic examination of allograft-biopsy specimens revealed extensive replication of the BK virus, necrosis of the cells of the tubules and collecting ducts, and varying degrees of interstitial inflammation.<sup>20</sup> Whereas biopsy of allograft tissue is mandatory for the definitive

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diagnosis of BK virus nephropathy, use of this invasive procedure seems inappropriate for monitoring. The detection of decoy cells by cytologic testing of urine, even in a quantitative manner (i.e., by cell counts), has a low positive predictive value (<20 percent in our series<sup>20</sup>) for BK virus nephropathy. In the current study, we investigated whether BK virus DNA can be detected by polymerase-chain-reaction (PCR) assay of the plasma of renal-allograft recipients with BK virus nephropathy.

## METHODS

### Subjects

We collected a total of 67 plasma or serum samples (hereafter referred to as plasma) for PCR amplification of BK virus DNA from nine renal-allograft recipients (two women and seven men) with histologically confirmed BK virus nephropathy. Four patients had received allografts from living donors and five had received allografts from cadaveric donors a mean ( $\pm$ SD) of  $46 \pm 28$  weeks before viral nephropathy was diagnosed. All nine patients had cells containing intranuclear viral inclusions ("decoy cells") in the urine at the time of diagnosis. None had ureteral stenosis. One patient had hypogammaglobulinemia, which was treated with intravenous immune globulin.

The initial immunosuppressive therapy in seven of the nine patients consisted of antilymphocyte globulin, cyclosporine, azathioprine, and methylprednisolone; in one patient it consisted of cyclosporine, mycophenolate mofetil, and methylprednisolone; and in one patient it consisted of tacrolimus, azathioprine, and methylprednisolone. Methylprednisolone was given initially (intravenously in a dose of 500 mg daily for three days), followed by oral prednisone (0.5 mg per kilogram of body weight daily; the dose was decreased by 5 mg every two weeks). The initial dose of azathioprine was 2 mg per kilogram daily, and it was decreased in steps of 25 mg. The dose of cyclosporine was guided by measuring trough blood concentrations, with a goal of 250 to 300 ng of cyclosporine per milliliter during the first 12 months and 150 to 200 ng per milliliter thereafter. Initially, allograft function in these nine patients was good, with serum creatinine concentrations of  $1.4 \pm 0.6$  mg per deciliter ( $125 \pm 56$   $\mu$ mol per liter) on day 6 after transplantation.

Subsequently, all nine patients had episodes of rejection, confirmed by biopsy, with rapid increases in serum creatinine concentrations that responded partially to the administration of intravenous methylprednisolone (500 mg daily) for three days and antilymphocyte preparations or muromonab-CD3 for six days. For seven of the eight patients whose initial immunosuppressive regimen did not include tacrolimus, high-dose tacrolimus was given as rescue therapy (trough blood concentration, 15 to 29 ng per milliliter), and for the eight patients who were taking azathioprine, the azathioprine was switched to mycophenolate mofetil (1000 mg twice daily). Renal function was progressively deteriorating in all nine patients when BK virus nephropathy was diagnosed. In three patients, nephrectomy of the allograft (performed 3, 20, and 72 weeks after the diagnostic biopsies) revealed persistence of the viral nephropathy. In two of the nine patients, the dose of the rescue immunosuppressive therapy was decreased to control the replication of virus. In one of these two patients, the serum creatinine concentration increased from 2.0 mg per deciliter (175  $\mu$ mol per liter) to 4.2 mg per deciliter (370  $\mu$ mol per liter), despite a change in immunosuppressive therapy, from tacrolimus and mycophenolate mofetil back to cyclosporine and azathioprine; when the dose of cyclosporine was reduced so that the trough blood concentration was about 150 ng per milliliter, histologic examination showed that the viral nephropathy had resolved, but the patient nonetheless had end-stage nephropathy involving the allograft and required hemodialysis. In the other patient, the tacrolimus used for rescue therapy was replaced by cyclosporine, but

the serum creatinine concentration rose to 2.1 mg per deciliter (185  $\mu$ mol per liter). When the dose of cyclosporine was lowered, the viral nephropathy was found to have resolved on histologic examination, and the serum creatinine concentration decreased to 1.5 mg per deciliter (135  $\mu$ mol per liter); the patient's regimen of mycophenolate mofetil (1000 mg twice daily) and prednisone (10 mg daily) remained unchanged.

We also collected 58 plasma samples for PCR amplification of BK virus DNA from 41 renal-allograft recipients (16 women and 25 men) who had no evidence of BK virus nephropathy. Sixteen of these patients were asymptomatic but were shedding decoy cells in their urine (3 women and 13 men; 7 had received allografts from living donors, and 9 had received allografts from cadaveric donors). In 8 of these 16 patients, examination of allograft tissue by light microscopy and immunohistochemical techniques revealed signs of acute rejection (in 2 patients) or chronic rejection (in 6 patients) but no viral inclusions. In the other 25 patients (13 women and 12 men; 13 had received allografts from living donors, and 12 had received allografts from cadaveric donors), no decoy cells were detected in the urine. An allograft-biopsy specimen from one of these patients showed signs of acute rejection but no signs of viral nephropathy. In 17 of these 25 patients, the results of cytologic tests of urine and PCR assays of plasma performed two to nine months later were available.

We also collected plasma samples for PCR amplification of BK virus DNA from 17 patients (6 women and 11 men) who had not undergone transplantation and who had HIV-1 infection (stage C3 according to the classification of the Centers for Disease Control and Prevention); these patients served as immunocompromised controls. Five had progressive multifocal leukoencephalopathy, four toxoplasmic encephalitis, two primary non-Hodgkin's lymphoma of the brain, one cytomegalovirus encephalitis, one myeloradiculitis, one peripheral neuropathy, and three HIV-1 encephalopathy. A study including 15 of these 17 patients has previously been published.<sup>23</sup>

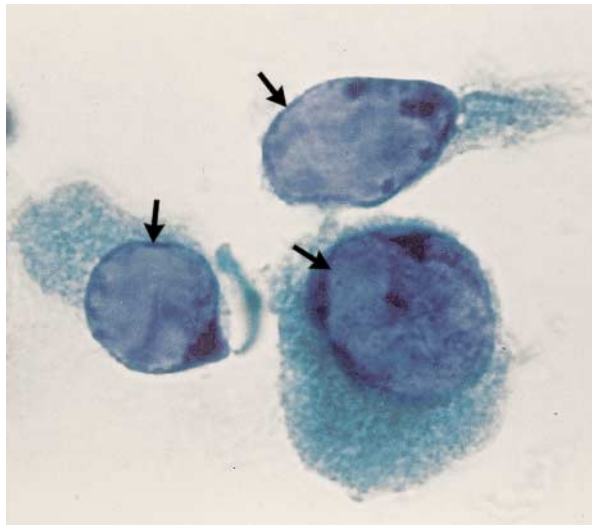
This retrospective study was performed in accordance with the guidelines of the University Hospitals Basel, Basel, Switzerland, and the protocol was approved by the ethics committee of Basel.

### Cytologic and Histologic Studies

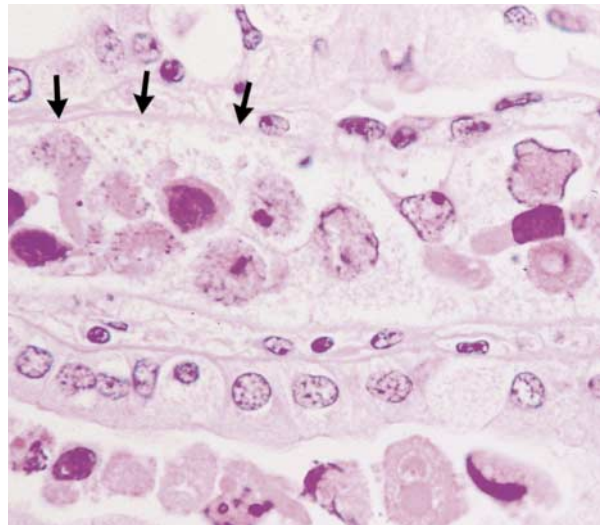
The cytologic and histologic procedures used to diagnose nephropathy due to infection with BK virus have been described previously.<sup>20</sup> Decoy cells were identified by their typical ground-glass intranuclear inclusions on cellular smears of urine samples stained by the Papanicolaou method (Fig. 1A). The number of decoy cells per 10 high-power fields was counted. Immunohistochemical detection of polyomavirus antigens was performed with mouse monoclonal antibodies against either the BK virus or the JC virus (Readysysteme, Bad Zurzach, Switzerland) and against SV40 large T antigen (Oncogene Research Products, Cambridge, Mass.), with use of 3-amino-9-ethylcarbazole as the chromogen and hematoxylin as the counterstain. On examination of the biopsy specimens, BK virus nephropathy was identified by the presence of intranuclear viral inclusion bodies in epithelial cells obtained from the entire length of the nephron. The cells were enlarged, often necrotic, and more abundant in distal tubular segments and collecting ducts (Fig. 1B).<sup>20</sup> There was no histologic, immunohistochemical, ultrastructural, or serologic evidence of concurrent infection with cytomegalovirus, Epstein-Barr virus, herpes simplex virus, or adenovirus in any of the patients.

### PCR Assays

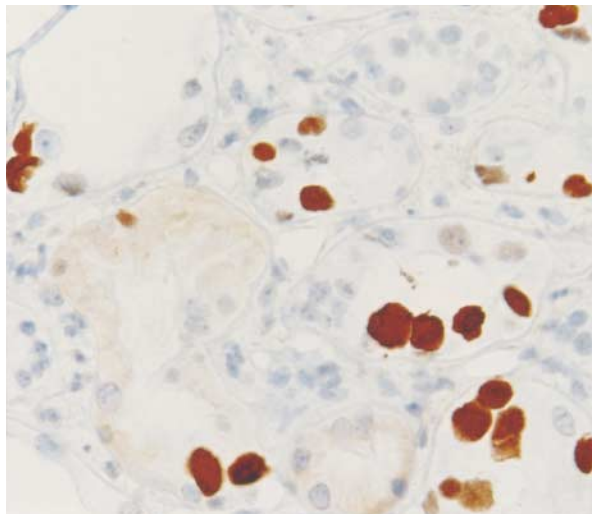
DNA was extracted from EDTA-anticoagulated plasma, serum, or urine by digestion with proteinase K and purification on silica columns before elution with double-distilled water, according to the protocol of the manufacturer of the kit (QIAmp, Qiagen, Basel). The use of semi-nested PCR assay for detection of polyomavirus DNA has been validated previously.<sup>20,23</sup> The outer primer pair 5'AAGTCTTTAGGGTCTTCTAC3' and 5'GTGCCAACCTATGGAACAGA3' was used to generate a common 176-bp am-



A



B



C

**Figure 1.** Urine and Allograft-Biopsy Specimens from a Patient with BK Virus Nephropathy.

Panel A shows the results of cytologic testing of urine. Decoy cells with characteristic ground-glass, intranuclear viral inclusion bodies (arrows) are visible (Papanicolaou stain,  $\times 400$ ). Panel B shows an allograft-biopsy specimen. Tubules with epithelial cells containing viral inclusions are visible. The BK virus causes nuclear enlargement and detachment of affected cells from the tubular basement membrane, leading to its denudation (arrows) (hematoxylin and eosin,  $\times 160$ ). In Panel C, immunohistochemical analysis of formalin-fixed, paraffin-embedded tissue reveals large T antigen in the nuclei of the epithelial cells of the tubules, thus confirming the diagnosis of BK virus nephropathy (mouse monoclonal antibody with 3-amino-9-ethylcarbazole as chromogen and hematoxylin as counterstain,  $\times 100$ ).

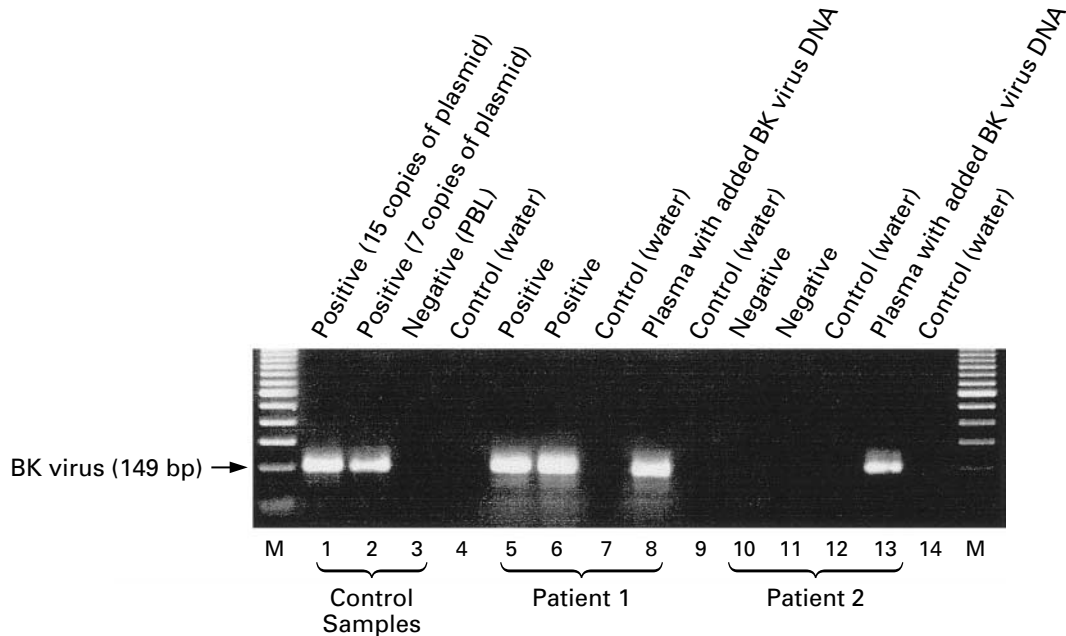
plicon. For specific detection, we used the common primer 5'AAG-TCTTAGGGTCTTCTAC3' with 5'GAGTCCTGGTGGAGTTC3' to obtain the 149-bp BK virus-specific fragment or with 5'GAATCCTGGTGGGAATACA3' to obtain the 146-bp JC virus-specific fragment. The threshold of detection was three to five copies of the BK virus and the JC virus plasmids per reaction. Each sample was analyzed in duplicate and with the addition of 15 copies of the respective plasmid to the reaction mixture to rule out inhibition of the PCR in negative results (Fig. 2). Quantification of the BK viral load in two patients revealed approximately 100,000 copies of the viral genome per milliliter of plasma collected at the time that BK virus nephropathy was histologically proved (unpublished data). The investigators performing the PCR analyses were blinded to other information about the patients (the immunosuppressive regimens, laboratory results, and results of histologic analysis).

### RESULTS

All nine renal-allograft recipients with BK virus nephropathy had the characteristic manifestations of this

infection, including high serum creatinine concentrations (a mean of  $3.8 \pm 2.1$  mg per deciliter [ $335 \pm 186$   $\mu\text{mol}$  per liter]), shedding of decoy cells in the urine, and extensive viral infection and necrosis of tubular cells in allograft-biopsy specimens (Table 1 and Fig. 1). In all nine patients, BK virus DNA, but not JC virus DNA, was detected in plasma samples drawn at the time that BK virus nephropathy was histologically proved (Table 1). For eight of the nine patients, the plasma sample had been drawn at the time of the initial diagnostic biopsy. For one patient, the only plasma sample available had been drawn at the time of nephrectomy of the allograft.

In contrast, BK virus DNA was detected in the plasma of only 1 of the 16 renal-allograft recipients who had decoy cells in the urine but no evidence of BK virus nephropathy (there was an absence of viral inclusions in renal-biopsy specimens in 8 of these patients). Among the 25 renal-allograft recipients with no decoy cells in the urine, BK virus DNA was detected in the plasma of 1 patient (Table 1). The allograft-biopsy specimen obtained at the same time as the plasma sample from this patient showed no signs



**Figure 2.** PCR Assay of BK Virus DNA in the Plasma of Patients with BK Virus Nephropathy.

Lanes 1 and 2 show positive controls with 7 and 15 copies of the BK virus plasmid, respectively; lane 3, a negative control (DNA from peripheral-blood leukocytes [PBL]); lanes 4, 7, 9, 12, and 14, negative controls (water); lanes 5 and 6, plasma positive for BK virus, in duplicate; lane 8, plasma to which 15 copies of BK virus plasmid were added; lanes 10 and 11, plasma negative for BK virus; and lane 13, plasma to which 15 copies of BK virus plasmid were added. M denotes molecular size marker.

of BK virus nephropathy. In 17 of these 25 patients, additional studies performed two to nine months later revealed no BK virus DNA in the plasma and no decoy cells in the urine. In the 17 patients with HIV-1 infection, no BK virus DNA was detected in plasma (Table 1). Thus, BK virus DNA was found in 2 of the 58 patients (75 samples) without histologic signs of BK virus nephropathy. During nine months of follow-up, no signs of graft dysfunction appeared in either patient.

Progressive impairment of renal function due to persistent BK virus nephropathy was documented by subsequent histologic evaluation of biopsy specimens and explanted graft tissue in eight of the nine patients. BK virus DNA was always detected in the plasma when persistent viral nephropathy was diagnosed by histologic examination of biopsy specimens and explanted grafts (Fig. 3). In five patients, viral DNA was no longer detectable in the plasma after these patients' immunosuppressive therapy was changed. In three patients, viral DNA became undetectable  $2 \pm 2$  weeks after allograft nephrectomy. In two patients, the doses of immunosuppressive drugs were reduced, in one because of end-stage nephropathy of the allograft and in the other as a first attempt to control the BK virus infection. It was consistent with the absence of BK virus DNA in the plasma that biopsy of

the patients' grafts at this time revealed signs of end-stage allograft nephropathy in the former patient and acute rejection in the latter but no further signs of BK virus nephropathy.

Plasma samples obtained 2 to 55 weeks before the histologic diagnosis of BK virus nephropathy were available for six of the nine patients. Viral DNA was not detected in any of the samples. In three of these six patients (Patients 4, 9, and 7), the time at which BK virus DNA was detected in plasma coincided with the histologic diagnosis of BK virus nephropathy. (The results for Patients 4 and 9 are shown in Fig. 3.) In the other three patients (Patients 2, 3, and 8), detection of BK virus DNA in the plasma preceded the histologic diagnosis by 16 to 33 weeks. In two of the nine patients, the first available plasma sample was collected at the time of diagnosis and was positive for BK virus DNA (data not shown). In one patient (Patient 5), the only available plasma sample had been obtained before transplantation, and it was negative for BK virus DNA (Fig. 3).

## DISCUSSION

Polyomavirus type BK has rarely been detected outside the urogenital tract, and its presence has often seemed clinically unimportant.<sup>7,12,24</sup> In the current study, we consistently detected BK virus DNA in the

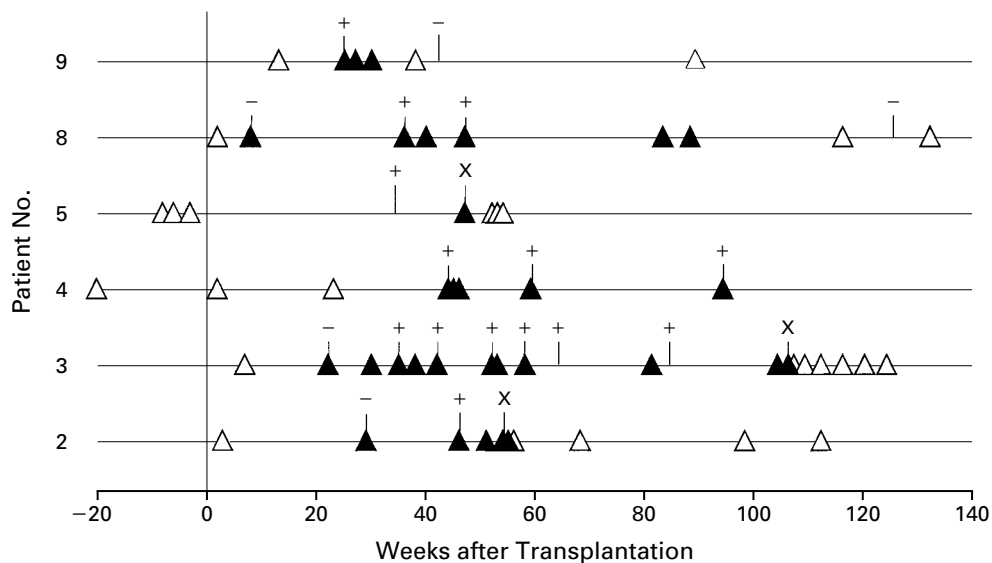
**TABLE 1.** DETECTION OF BK VIRUS DNA BY PCR IN THE PLASMA OF PATIENTS WITH BK VIRUS NEPHROPATHY AND CONTROL SUBJECTS WITH HIV-1 INFECTION.\*

PATIENTS	AGE	DECOY CELLS IN URINE	BK VIRUS IN PLASMA BY PCR	TIME TO DIAGNOSIS AFTER TRANSPLANTATION†	SERUM CREATININE‡
	yr	no. of patients/no. tested		wk	mg/dl
Renal-allograft recipients					
With BK virus nephropathy and with +++ decoy cells in urine (n=9)	51±10	9/9	9/9	46±28 (36; 18–107)	3.8±2.1 (2.6; 1.8–5.9)
Without BK virus nephropathy but with decoy cells in urine (n=16)	51±12	16/16	1/16	37±29 (30; 7–103)	1.6±0.6 (1.4; 0.9–2.0)
+++ Decoy cells		7/16	1/7		
++ Decoy cells		4/16	0/4		
+ Decoy cells		5/16	0/5		
Without decoy cells in urine (n=25)	52±12	0/25	1/25	30±16 (26; 10–60)	1.7±1.4 (1.2; 0.6–7.0)
Patients with HIV-1 infection (CDC stage C3) (n=17)	34±6	ND	0/17	NA	1.0±0.3 (1.0; 0.6–1.2)

\*Plus-minus values are means ±SD. Plus signs denote the number of decoy cells per 10 high-power fields (+, one per 10 high-power fields; ++, two to five per 10 high-power fields; +++, more than five per 10 high-power fields). PCR denotes polymerase chain reaction, CDC Centers for Disease Control and Prevention, ND not done, and NA not applicable.

†Values in parentheses are medians and ranges.

‡Values in parentheses are medians and ranges. To convert values for serum creatinine to micromoles per liter, multiply by 88.4.



**Figure 3.** Time Course of Detection of BK Virus DNA in the Plasma of Representative Renal-Allograft Recipients with BK Virus Nephropathy.

Results of PCR assays for BK virus DNA in plasma are plotted for representative patients as a function of time after transplantation. Solid triangles represent positive results on PCR; open triangles, negative results on PCR; plus signs, the presence of BK virus nephropathy on allograft biopsy; minus signs, the absence of BK virus nephropathy on allograft biopsy; and X, allograft nephrectomy. All the explanted kidneys had histologic evidence of BK virus nephropathy.

plasma of renal-allograft recipients with BK virus nephropathy — a finding with diagnostic, therapeutic, and pathogenetic implications. Formerly an exceedingly rare condition, BK virus nephropathy has been increasingly identified as a cause of dysfunction and loss of renal allografts. Among the suggested risk factors for this infection are recurrent episodes of rejection and intensive immunosuppressive regimens with new drugs such as tacrolimus and mycophenolate mofetil.<sup>18</sup> A systematic study of allograft-biopsy specimens and allograft tissue after nephrectomy revealed that tubular necrosis is the chief cause of graft dysfunction and that it is a direct consequence of extensive replication of the BK virus.<sup>20</sup> No antiviral treatment for this infection is currently available, and the only therapeutic approach seems to be a careful reduction in the doses of immunosuppressive therapy to control the replication of BK virus.<sup>1,19</sup>

With respect to diagnosis, we found that PCR assays for BK virus DNA in plasma had a sensitivity of 100 percent and a specificity of 88 percent for detecting BK virus nephropathy when we included only patients with histologically confirmed diagnoses (positive predictive value, 82 percent; negative predictive value, 100 percent). The specificity was 95 percent when all 41 renal-allograft recipients who did not have evidence of BK virus nephropathy were included in the analysis. Among all 58 control patients, only 2 patients with no evidence of BK virus nephropathy had plasma samples that were positive for BK virus DNA; 1 of them had acute rejection and the other had chronic rejection. Review of their medical records revealed that both patients had a history suggestive of reactivation of the BK virus, including repeated episodes of rejection, ureteral stenosis in the graft (in one patient), and an immunosuppressive regimen that included mycophenolate mofetil (in the other patient).

With respect to the course of BK virus nephropathy, we found that the presence of viral DNA in plasma reflected the dynamics of the disease: the conversion of plasma from negative to positive for BK virus DNA after transplantation, the presence of DNA in plasma in conjunction with the persistence of viral nephropathy on histologic examination, and the disappearance of viral DNA from plasma after the doses of immunosuppressive therapy had been reduced.

How does the BK virus gain access to the bloodstream? From our data and a review of the literature,<sup>1,8,14-16,20</sup> it is evident that replication of the BK virus, even when it leads to abundant shedding of decoy cells in the urine, is not necessarily associated with viral nephropathy or with the presence of BK virus in plasma. This idea is consistent with the presumed urothelial origin of decoy cells and the detection of virus-infected cells primarily in superficial urothelial layers, from which the BK virus is unlikely to reach the circulation. However, after cell-to-cell

spread from renal papillae to terminal collecting ducts,<sup>20</sup> the BK virus may reach the circulation by way of the adjacent peritubular capillaries. This hypothesis may explain the early detection of BK virus DNA in the plasma of three patients whose biopsy specimens were initially negative for the virus.

Although prospective studies are needed for further validation, our data provide evidence that the detection of BK virus DNA in the plasma identifies renal-allograft recipients who are at risk for BK virus nephropathy. Among patients with impaired graft function, we currently screen for decoy cells in urine by cytologic tests. If decoy cells are present, we perform PCR analysis specific for the BK virus in plasma samples; if the samples are positive for the virus, we confirm the diagnosis by allograft biopsy and perform additional PCR assays on plasma to monitor the course of the nephropathy.

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