Use of hybridot assay to screen for BK and JC polyomaviruses in non-immunosuppressed patients

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SUMMARY Urine samples from 50 patients attending a genitourinary outpatient clinic and from 13 renal allograft recipients were investigated for evidence of infection with human BK and JC polyomaviruses using cytology and a new DNA hybridot assay. Forty per cent of samples from the renal allograft recipients were positive by cytology and 75% by DNA hybridisation, indicating that hybridot assay is more sensitive than cytological screening. BK and JC viral DNA was found in 20% of the patients attending the genitourinary clinic, showing infection with BK virus and JC virus in a group of patients with clinical conditions not normally associated with immunological deficiency—a finding that has not been reported before.

BK virus (BKV) and JC virus (JCV) are members of the polyoma subgroup of papovaviruses and their natural host is man. Although seroepidemiological studies show that most people have been exposed to both viruses in early childhood,1-3 replication and excretion of BKV and JCV have been detected only in patients whose immune reaction is impaired by treatment or disease,4-5 and in pregnant women.6 In most cases virus activity is manifest as asymptomatic viruria, although an association with haemorrhagic cystitis7-9 and ureteric obstruction10 has been reported. Active infection with JCV is also known to cause the rare demyelinating disease, progressive multifocal leucoencephalopathy, although the mechanism of cerebral infection is not known.11 12

Because virus culture is slow,4 detection of virus excretion in allograft recipients and other immunosuppressed patients has relied largely on cytological examination of urinary sediment.5 13 14 Large inclusion bearing transitional cells can be seen on light microscopic examination of Papanicolaou stained smears prepared from the urine of patients with an active infection. This approach is quick and can be used for screening but suffers from the disadvantage that it only provides indirect evidence of infection, which must be confirmed by conventional virological methods.

A method of detecting BKV and JCV in urine using DNA probes specific for the viral nucleic acids has been described.15 This technique has the advantage over cytological methods of detection in that it provides objective evidence of polyomavirus infection and permits immediate identification of virus type. We used a modification of this DNA technique to screen for BKV and JCV in renal allograft recipients and patients attending a genitourinary outpatient clinic, and compared the sensitivity of this approach with cytological investigation of the same urine sample.

We report for the first time the presence of polyomavirus DNA in the urine of patients who were not receiving cytotoxic treatment and who were not considered to be immunocompromised by drugs or disease.

Patients and methods

GROUP I

Fifty patients (30 men and 20 women aged 19-87 years) attending the genitourinary outpatient clinic at St Mary’s Hospital were included. Forty six of the patients presented with genitourinary disease and the remaining four were asymptomatic and attending follow up after treatment. The conditions studied included: urinary tract infection (n = 13), prostatic hypertrophy (n = 7), renal calculi (n = 3), urethral stricture (n = 4), bladder cancer (n = 2), stress incontinence (n = 4), other (n = 3). In 14 cases the cause of the symptoms were unknown: this included eight patients with haematuria. None of the patients was receiving or had received cytotoxic or immunosuppressive treatment.

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GROUP 2
This group comprised 13 renal allograft recipients (eight men, five women, age range 25–63 years) attending the renal outpatient clinic at St Mary's Hospital for a routine health check. All were receiving immunosuppressive treatment (prednisolone 5–15 mg daily, azathioprine 75–200 mg daily).

CONTROLS
Case RS, a known virus excretor and a healthy male doctor aged 61, was found by chance to be excreting polyomavirus on cytological examination of a sample of urine in July 1979. Cytological examination repeated annually ever since has shown inclusion bearing cells in the smears of the urine sediment. Attempts at virus isolation in 1980 and 1983 were negative but electron microscopy of inclusion bearing cells in the same urine samples showed polyomavirus particles in the nuclei of the cells. Haemagglutination inhibition tests performed by Dr S D Gardner on blood samples collected in August 1979, February 1980, and April 1983 showed BK antibody titres of 320, 160, and 140; and JCV antibody titres of 640, 1280, and 1280, respectively. Immunological profiles were investigated at the same time as the urine samples were collected. A borderline subnormal lymphocyte response to phytohaemagglutination stimulation was recorded in February 1980, July 1980, and March 1981, and a slightly reduced total T cell count in April 1982. Both results were regarded as secondary to infection with these viruses, an interpretation borne out by the facts that all tests on April 1983 yielded normal results.

Cell culture fluid (2 ml) containing BKV was diluted in phosphate buffered saline (dilution range: 0–1/4000) and 400 μl aliquots were spotted on to the Zetaprobe membrane filters. The concentration of viral particles/ml of culture fluid was determined by electron microscopy and the sensitivity of the BKV DNA probe evaluated.

Fresh mid stream urine (10–40 ml) was collected into a universal container and aliquoted for cytological and microbiological studies and for DNA hybridisation.

CYTOLOGY
Urine (5–20 ml) was filtered through a Metricel membrane filter, pore size 5 μm, diameter 45 mm, vacuum pressure not exceeding 5 mm Hg. The filters were fixed in 95% alcohol, stained by the Papanicolaou method, and examined by light microscopy for the presence of intranuclear inclusion bodies.

MICROBIOLOGY
The Mast bacteruria dipstick test was used. A fixed volume of urine was spread on to culture plates containing cystine, lactose, electrolyte deficient agar, a medium which supports a wide range of bacteria, and incubated at 25°C for 24 hours. The plates were then examined for growth of bacteria. Twenty bacterial colonies are equivalent to 10⁵ organisms/ml.

DNA HYBRIDISATION
Three DNA probes were used. The BKV probe was constructed from the whole genome of pBK (Dun) and the JCV probe from the whole genome of pJCV (Mad-1). Both were cloned into the Barn HI site of the pBR 322 Escherichia coli plasmid and radioactively labelled to a specific activity of at least 10⁷ dpm/μg with ³²P by nick translation. The third probe, prepared in the same way, was the pBR322 plasmid alone.

The unfiltered urine samples were dotted on to a Zetaprobe filter using a Biorad hybridot apparatus. Aliquots of urine (400 μl) were immersed in boiling water for five minutes and quenched on ice for two minutes before being transferred into the wells of the manifold. Suction was applied to adsorb the DNA on to the filters. The filters were air dried and baked for three hours at 80°C. Before hybridisation the filters were first treated by overnight incubation at 65°C in a solution of 0.1 × sodium chloride, sodium citrate, 0.05% sodium dodecyl sulphate, 5 × Denhard’s solution, containing 100 μg/ml sonicated, denatured salmon sperm DNA. The filters were then incubated overnight at 42°C in a prehybridising solution of 50% deionised formamide, 0.05% sodium dodecyl sulphate, 80 mM Tris-hydrochloric acid (pH 7.8), 4 mM edetic acid, 0.75 M sodium chloride, to which was again added 100 μg/ml of the salmon sperm DNA. The filters were then hybridised overnight at 42°C in a 1–2 ml of the prehybridising solution containing 2 × 10⁶ dpm/ml heat denatured probe.

After hybridisation the filters were washed for three hours in a solution containing 0.1 sodium chloride, sodium citrate, and 1% sodium dodecyl sulphate at 65°C. After drying the filters were autoradiographed for 56 hours on preflushed Kodak Exomat film at –70°C.

The figure shows a filter after hybridisation with BKV DNA probe: seven of the 35 samples on the filter are positive.

RESULTS

GROUP I
A urine sample from each of the 50 patients attending the genitourinary clinic was investigated for bacterial content and by DNA hybridisation. Thirty three of these samples were also investigated cytologically. No inclusion bearing cells were seen on cytological examination.

Microbiology A significant bacteriological growth
hybridisation. Seven samples from four patients were positive by cytology (44%), and 12 samples from eight patients were positive by DNA hybridisation (75%).

Inclusion bearing transitional cells were seen in seven samples. Of these, four contained DNA sequences which hybridised to both BKV and JCV probes; one contained sequences that hybridised to the BKV probe alone and one with JCV probe alone. The remaining sample showed no evidence of hybridisation with either probe.

Nine urine samples contained no inclusion bearing transitional cells on cytological examination. Of these, one sample contained DNA sequences that hybridised to both BKV and JCV probes; five contained sequences that hybridised with JCV probe alone, and the remaining three samples were negative.

**Controls**

**Case RS** One urine sample taken in August 1985 was investigated by cytology and DNA hybridisation. Inclusion bearing transitional cells were seen, and DNA sequences homologous to both BKV and JCV DNA were found.

**BKV culture fluid** The concentration of BKV particles in the fluid was calculated to be $17 \times 10^6$/ml. Hybridisation of 400 µl aliquots of culture fluid with BKV DNA probe was detected at 1/100, with the DNA probe radiolabelled with $^{32}$P to a specific activity of $2 \times 10^8$ dpm/µg DNA, indicating that hybridisation could detect a minimum of $7 \times 10^4$ viral particles per sample.

**pBR 322 probe** No hybridisation by any of the samples was detected.

**Discussion**

By using a hybridot assay for BKV and JCV as a screening tool, we were able to show the presence of viral DNA in the urine of 62% of renal allograft recipients and 20% of patients attending a genito-

**Table**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Clinical findings</th>
<th>DNA hybridisation</th>
<th>Microbiology</th>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>65</td>
<td>Peyronies syndrome</td>
<td>BKV + JCV -</td>
<td>No growth</td>
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<td>2</td>
<td>F</td>
<td>76</td>
<td>Renal calculi, follow up after treatment</td>
<td>BKV + JCV -</td>
<td>No growth</td>
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<tr>
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<td>M</td>
<td>51</td>
<td>Haematuria, cause unknown</td>
<td>BKV + JCV +</td>
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<td>83</td>
<td>In situ carcinoma of bladder</td>
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</tr>
<tr>
<td>5</td>
<td>M</td>
<td>78</td>
<td>Incontinence following TURP for prostatic hypertrophy</td>
<td>BKV + JCV -</td>
<td>Mixed flora</td>
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<tr>
<td>6</td>
<td>M</td>
<td>67</td>
<td>Haematuria, cause unknown</td>
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<td>No growth</td>
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<tr>
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<td>M</td>
<td>57</td>
<td>Prostatic hypertrophy</td>
<td>BKV + JCV +</td>
<td>No growth</td>
</tr>
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</table>

Mean age of DNA hybridising group = 64.2 years ($n = 10$; SD = 14.52).

Mean age of non-DNA hybridising group = 55.4 years ($n = 40$; SD = 17.98).

TURP = Transurethral resection of prostate.
urinary clinic. Cytological examination of urine sediment from both groups of patients indicated virus in the transplant group alone. Our study has shown that viral DNA may be found in the urine of immunosuppressed patients in the absence of cytological evidence of infection.\textsuperscript{5, 13, 17} We have also shown that viral DNA may be found in the urine of patients with clinical disorders not usually associated with immunological deficiency, an observation that has not been reported before, as far as we know. Furthermore, a healthy male doctor had had asymptomatic viruria confirmed by cytology and electron microscopy for many years with no evidence of immunological impairment, thereby supporting the hypothesis that replication and excretion of human polyomaviruses depends on a variety of factors, only one of which is associated with immunocompetence.

Correlation of the microbiological findings with the results of hybridisation of the 50 patients attending the genitourinary clinic showed no association between the presence of bacterial urinary tract infection and BK or JC viruria. As there have been several reports associating BKV and JCV excretion with haemorrhagic cystitis\textsuperscript{7-9} and ureteric obstruction,\textsuperscript{10} we examined the relation between the clinical condition of the patients in group 1 and the presence of viral DNA. The urine of two of eight patients with unexplained haematuria, three of 11 patients with evidence of obstruction to urinary flow (four urethral stricture, seven prostatic hypertrophy), one of three patients with renal calculi, one of two patients with a history of bladder cancer, and three other patients with cystocele, suprapubic pain, and epididymitis hybridised to the DNA probes. This indicates that the presence of viral DNA is associated with a variety of urinary tract diseases. BKV and JCV have been detected in up to 30\% of cadaveric kidneys obtained at necropsy from patients who were otherwise free of renal disease.\textsuperscript{18} The type of kidney cell harbouring the viral genome, however, has not been identified.\textsuperscript{18} Our study suggests that the viral DNA detected in the urine was in transitional epithelium as it is very rare to find cells from the renal parenchyma or medulla in the urine. The association of BKV and JCV and haemorrhagic cystitis may be related to copy number, and we propose to investigate this aspect of infection using the quantitative assay described in this paper.

Our study shows that the hybridot assay is a sensitive screening tool that can be used to process large numbers of urine samples; only a very small sample of fresh or frozen urine is required. The use of a control sample containing a known concentration of BKV permitted estimation of the sensitivity of DNA hybridisation and comparison with cytology and virus isolation. Hybridisation was observed when the control sample contained a minimum of $7 \times 10^4$ viral particles. In contrast, an inclusion body in the nucleus of a polyomavirus infected cell contains $10^7$ viral particles.\textsuperscript{19} Hence the hybridot assay can detect polyomavirus in urine samples that would be regarded as negative by light microscopy. This has been clearly shown in our study. We found that 31\% of the immunosuppressed patients were excreting virus cytologically, whereas the hybridot assay detected polyomavirus DNA in 62\% of the patients.

The specificity of the test has been established by the use of the culture fluid infected with BK tissue and case RS, a known polyomavirus excretor, as controls. In addition, the pBR 322 plasmid alone was used in a control hybridisation test to determine whether any of the positive results were due to bacterial contaminants in the sample hybridising to any pBR322-related sequence. No hybridisation was observed, confirming the specificity of the BKV and JCV probes. BKV or JCV DNA, or both, were detected in all but one of the specimens that were cytologically positive. In this case inclusion bearing cells were present, although the sample did not hybridise to the probe. The inclusion bearing transitional cells were probably caused by cytomegalovirus infection, which is common in transplant patients. Light microscopy has shown that the cellular changes induced by the two viruses closely resemble each other,\textsuperscript{14} and illustrates the limitation of cytology.

The sensitivity and specificity of the hybridot technique makes it a valuable tool in clinical practice and research. The technique can be adapted for use on tissue samples and would be particularly appropriate for the investigation of patients with symptoms and signs suggestive of progressive multifocal leucoencephalopathy. It could also be used to investigate primary infection in children. Although there is indirect evidence of an association with respiratory tract infection,\textsuperscript{20} the portal of entry and the clinical manifestations of primary infection are still not known, and the technique could be readily modified for hybridisation of nasal mucous or throat swabs for large scale epidemiological studies.

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Screening for BK and JC viruses in non-immunosuppressed patients

References


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