Diagnosis of childhood BK virus cystitis by electron microscopy and PCR

K Saitoh, N Sugae, N Koike, Y Akiyama, Y Iwamura, H Kimura

Abstract
A case of BK virus cystitis in a 5 year old boy is reported. This patient, who was not immunocompromised, had had acute cystitis for two weeks. Many intracytoplasmic inclusions were observed in urinary sediment smears stained by the Papanicolaou method. Electron microscopic examination showed virus particles, presumed to be human polyomavirus, in the nuclei of the degenerated urothelial cells. A DNA sequence of the BK virus was detected in 200–300 urothelial cells in Papanicolaou stained smears by the polymerase chain reaction. BK virus is an unusual cause of symptomatic cystitis in a healthy child.

Human polyomaviruses are often detected in the urine of patients receiving chemotherapy for carcinomas, as well as in the urine of organ transplant recipients who are receiving immunosuppressive treatment.1-2 Two major strains of human polyomaviruses, BK virus and JC virus, have been identified.3-4 BK virus is known to cause haemorrhagic cystitis and JC virus is detected in the brain tissue of immunologically impaired patients with progressive multifocal leucoencephalopathy. It is widely believed that these viruses rarely affect non-immunocompromised subjects. BK and JC viruses are, however, sometimes detected in the urine samples of healthy people without any clinical manifestations, especially in pregnant women and older people.5-6

Case report
A 5 year old boy presented with urinary frequency and abdominal pain during urination. He was not immunocompromised and had no history of urinary tract problems. He was afebrile and had no other symptoms. The urine was clear with a pH of 5-5 and specific gravity of 1.025. Routine urinalysis performed on the third- and fifth-day urines showed 5–8 erythrocytes and white cells, and 10–20 urothelial cells per high power field. The urothelial cells in the urinary sediments prepared by the Papanicolaou staining method were degenerate and some had large and hyperchromatic nuclei. These were assumed to be cells infected with virus, although viral culture and serological examination for virus infection was not performed. He had not received antibiotics, and urine culture for bacteria identified no causative organism. The clinical symptoms decreased gradually and the abnormal cells in the urine disappeared two weeks later. The blood samples indicated no abnormalities.

Methods
Cytological smears were prepared from urinary sediment obtained on the third, fifth, and 15th days. The smears were fixed in 95% ethanol and stained by the Papanicolaou method.

The third day urinary sediment was examined electron microscopically. The cell pellet was fixed with 2.5% glutaraldehyde and 1% osmic acid, dehydrated with graded alcohol, and embedded in Polybed 812. Ultrathin sections were made, stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7000, Hitachi, Japan).

About 200–300 abnormal urothelial cells on a glass slide prepared by the Papanicolaou method were used for DNA extraction. The Papanicolaou stained smear was immersed in xylene in a 60°C incubator for one day, after which the cover glass was detached. Xylene was removed with alcohol. After washing with distilled water, digestion of the protein of the urothelial cells was performed on the glass slide in 300 μl proteinase K solution (200 μg/ml) at 37°C for 18 hours. The DNA was then extracted with phenol. Extractions with chloroform were successively repeated three times and the phenol was eliminated. Next, the RNA in the sample was digested with 100 μg/ml RNase at 37°C for 15 minutes and the DNA was again extracted with phenol. Extractions with chloroform were then repeated three times and the RNase was completely eliminated. The DNA sample was finally precipitated with ethanol and dissolved in 50 μl of 10 mM TRIS-HCl buffer (pH 7-5) containing 1 mM EDTA. The sample was frozen and thawed several times before PCR to destroy inhibitors of Taq polymerase in the urine.7

The sample DNA, the sample DNA diluted three times with phosphate buffered saline (PBS), DNAs of BK and JC viruses used as positive controls and human placental DNA used as a negative control, and PBS without DNA were prepared for amplification by polymerase chain reaction (PCR). The control BK virus DNA used had been extracted from the urine of a patient with a urinary bladder carcinoma and was found to be positive for BK virus using PCR. The JC virus DNA was extracted from the brain tis-
issue of a patient with adult T cell leukaemia, complicated with progressive multifocal leukoencephalopathy at the terminal stage, and was found to be JC virus positive by in situ hybridisation and PCR. A single pair of 20-base oligomer primers, PEP-1 and PEP-2, were used to amplify the homologous nucleotide sequences of the BK and JC viruses.

Sample and control DNAs were amplified in a DNA thermal cycler (Thermal Sequencer TSR-300, Iwaki Garasu, Japan) in 100 μl solutions containing either 10 μl of target DNAs or 10 μl of controls, 0.5 μM of the pair of primers, 2.5 U of Taq polymerase (AmpliTaq, Takara Shuzo, Japan) and 10 × reaction buffer (50 mM KCl, 10 mM TRIS-hydrochloride (pH 8.3), 1.5 mM MgCl₂, and 0.01% weight/volume gelatin). A thermal cycle consisted of step one: denaturing of double stranded DNA at 94°C for 70 seconds; step two: annealing of the primers at 55°C for 60 seconds; step three: primer extension at 72°C for 20 seconds. A 5 minute denaturing step at 94°C was included in the first cycle. Forty cycles of amplification were performed.

Electrophoresis of 10 μl of the PCR products from the sample DNA and controls was performed on 3.5% agarose gels. Digestion with BamHI restriction endonuclease (Nippon Gene, Japan) was performed before electrophoresis for discrimination between BK and JC viruses DNA sequences. The gels were stained with ethidium bromide and the amplified DNA sequences were visualised as bands of appropriate sizes with ultraviolet fluorescence.

**Results**

Urothelial cells in the third- and fifth-day Papanicolaou stained specimens were noticeably degenerate, and some had intranuclear inclusions with a diameter in the range of 15–30 μm (fig 1). The nucleus was dark and swollen but homogeneous and the nuclear membranes were smooth. The nucleoli were inconspicuous and neither nuclear pleomorphism nor irregular indentations were evident. The cytoplasm of most of the inclusion bearing cells was narrow and phagosomes granules were not observed.

Many virus particles were observed in the nuclei of the degenerate urothelial cells at electron microscopy (fig 2). The virus particles were round and uniform with a diameter in the range of 35–45 nm. There were no envelopes covering the particles. The particles were often densely packed in the nuclei, but sometimes were linearly aligned, branching irregularly and displaying arabesque patterns. A few crystal structures were observed.

DNA extracted from the abnormal urothelial cells and the control BK and JC virus DNA sequences was amplified by PCR. The sample and BK virus DNAs were not cleaved by BamHI and showed a single band at 176 base pairs (fig 3). The control JC virus DNA was also amplified, but the PCR product was cleaved by BamHI and formed two bands at 120 and 53 base pairs.

**Discussion**

Human polyomaviruses are small non-enveloped DNA viruses that are classified into two main strains, BK and JC. Human polyomaviruses are ubiquitous and people are often infected in infancy and early childhood. Infection is asymptomatic in most cases, and many people are reported to have anti-human polyomaviruses antibodies without an associated illness. After the primary infection human polyomaviruses are presumed to remain latent in the kidneys. Immunosuppressive medication taken for renal or bone marrow transplantation, chemotherapy used to treat malignant tumours, autoimmune diseases and diabetes mellitus may all reactivate human polyomaviruses and cause haemorrhagic cystitis or progressive multifocal leucoencephalopathy. It is very unusual for human polyomaviruses, both BK and JC, to infect small children with healthy
immune systems and to cause clinical illness: only two cases of human polyomaviruses cystitis in healthy children have been reported.10

Although urinary tract infection by human polyomaviruses is generally caused by BK virus, JC virus has also been detected in some of the urine samples of bone marrow transplant recipients and healthy subjects.3-6

Cytological examination of urine samples is convenient for the primary screening of human polyomaviruses infection. BK-virus infected urothelial cells have been reported to have large and hyperchromatic intranuclear inclusions, and the abnormal urothelial cells of the present case were consistent with those of the reported cases.

Electron microscopic examination can show the shape and size of the virus particles and distinguish the groups of viruses.7 The ultrastructural features of the virus particles of the present case-round, non-enveloped, and with a diameter in the range of 35-45 nm corresponded to those of human polyomaviruses particles described in the previous reports. It was difficult, however, to identify the strain using morphological methods. We could not determine, therefore, whether the aetiological agent was BK or JC virus using either cytology or electron microscopy.

PCR was developed for the analysis of DNA sequences in tiny quantities of cells. PCR is effective for the definitive diagnosis of strains of the human polyomaviruses group.4

In the present study we used the small DNA sequences of human polyomaviruses, PEP-1 and PEP-2, as the oligomer primers. The DNA sequences of BK and JC viruses resemble each other and PEP-1 and PEP-2 are complementary to the DNA sequences of BK and JC viruses. Therefore, this single set of primers can amplify the nucleotide sequences of both BK and JC virus DNA with the aid of Taq polymerase in the PCR cycle. The length of the amplified BK and JC virus DNA fragments is reported to be 176 and 173 base pairs, respectively. The amplified JC virus sequence contains a BamHI site, and is expected to be cleaved into fragments of 120 and 53 base pairs by BamHI. Conversely, the amplified BK virus sequence does not contain a BamHI site and forms a single band at 176 base pairs. The amplified sample DNA of the present case formed a single band at 176 base pairs, and the virus in the abnormal urothelial cells was shown to be BK virus.

The duration of the clinical illness, frequency, pain at urination and mild haematuria, and the appearance of the degenerate urothelial cells containing intranuclear inclusions in the urinary sediment were almost the same in the present case. The cytological findings of the urothelial cells in the Papanicolaou stained smears and the ultrastructural characteristics of the virus particles in the nucleus were suggestive of human polyomaviruses infection. Electron microscopically the urothelial cells containing the virus particles were also severely degenerate, which suggested that the virus had cytopathic effects. Moreover, we identified the nucleotide sequence specific to BK virus by analysing the abnormal urothelial cells of the urinary sediment using PCR. Consequently, this 5 year old boy was definitively diagnosed as having acute BK virus cystitis.