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A Sundsfjord, A R Spein, E Lucht, T Flaegstad, O M Seternes and T Traavik

Detection of BK Virus DNA in Nasopharyngeal Aspirates from Children with Respiratory Infections but Not in Saliva from Immunodeficient and Immunocompetent Adult Patients

ARNFINN SUNDSFJORD,1,2* ANNA-RITA SPEIN,1 ERIK LUCHT,3,4 TROND FLÆGSTAD,1,5 OLE MORTEN SETERNES,1 AND TERJE TRAAVIK1

Department of Virology,1 Department of Microbiology,2 and Department of Pediatrics,5 School of Medicine, University of Tromsø, Tromsø, Norway, and Department of Virology, National Institute for Infection Control,4 and Dental Clinic of Infectious Diseases, Departments of Microbiology and Oral Surgery, Huddinge University Hospital,3 Karolinska Institute, Stockholm, Sweden

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Our understanding of important stages in the pathogenesis of the human polyomavirus BK virus (BKV) and JC virus (JCV) infections is limited. In this context, nasopharyngeal aspirates from 201 children with respiratory diseases and saliva from 60 healthy immunodeficient adults and 10 healthy control females were collected and analyzed for the presence of BKV and JCV DNA by PCR. Neither BKV nor JCV DNA was detected in the saliva specimens. We demonstrated BKV DNA, but no infectious BKV, in 2 of 201 nasopharyngeal aspirates. Each sample contained a single rearranged noncoding control region variant of BKV. The results indicate that (i) BKV and JCV are not regularly associated with respiratory infections in children requiring hospitalization, (ii) nasopharyngeal cells are not an important site for primary replication of human polyomaviruses BKV and JCV, and (iii) the salivary glands and oropharyngeal cells seem not to be involved in BKV and JCV persistence. We propose that for the polyomaviruses BKV and JCV the alimentary tract should be considered as a portal of entrance to the human organism.

The human polyomaviruses (PyVs) BK virus (BKV) and JC virus (JCV) have independent and worldwide distributions in the human population (6, 37). The two primary isolations of BKV and JCV were described in 1971 (17, 30). Both viruses establish persistent infections following primary infections, which are mainly acquired subclinically during childhood (6, 16, 48). The brain and kidneys seem to be included as target organs for latency (3, 4, 10, 13, 34, 40, 48, 49, 51, 55). Several studies have supported a general impression that reactivation of latent BKV and JCV infections is associated with immunological impairment (2–4, 9). However, recent reports have provided results indicating a high prevalence of JCV viruria in immunocompetent individuals, while BKV viruria seems to be associated with immunodeficiency (22, 34, 48). Pathogenetic effects of PyV reactivations have been reported in immunosuppressed patients (3, 9, 54), but progressive multifocal leukoencephalopathy is so far the only clinical illness conclusively associated with a human PyV (JCV).

The portals of entry, the target cells for primary replication, and all the harbor cells for latent BKV and JCV genomes have yet to be identified. BKV seroconversions in children may be accompanied by both tonsillitis and upper respiratory infections (18, 19, 32), and results of seroepidemiological studies are consistent with infections acquired through the respiratory tract. To obtain more knowledge about these basic pathogenetical aspects, saliva from immunodeficient and immunocompetent adults and nasopharyngeal (NP) aspirates from hospitalized children with respiratory infections were collected and analyzed for the presence of viral DNA by PCR.

Origins of the samples. Unstimulated whole saliva was collected from 60 healthy immunodeficient virus type 1 (HIV-1)-infected adults and from 10 healthy adult controls attending the Dental Clinic of Infectious Diseases, Huddinge University Hospital, Karolinska Institute (29, 30). The 60 HIV-positive persons were classified according to the Centers for Disease Control (CDC) classification (CDC 1986), with 20 each in groups II, III, and IV (7).

NP aspirates were collected from hospitalized children with respiratory infections, 0 to 5 years of age, at the Department of Pediatrics, University Hospital of Tromsø, primarily for the detection and recovery of respiratory syncytial virus (RSV) and parainfluenza viruses (24, 26). A 0.5- to 1-ml aliquot of each processed specimen was stored in cell culture medium at −70°C until further analysis.

Conditions for PCR analysis and identification of PCR products. PCR amplifications were performed in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, Calif.). Precautions cited by Kwok were followed (25). Standard PCR conditions with 0.25 μM each primer were used unless otherwise indicated. For each PCR product an aliquot of 15 μl was analyzed on ethidium bromide-stained 2 or 3% agarose gels. All experiments were conducted in parallel with positive and negative controls.

For the detection of T-antigen coding sequences the PyV primer pair was used (5). Furthermore, the earlier described BKTT primers flanking the noncoding control region (NCCR) of BKV and JCV were used (14, 48, 49). In addition, a new primer, GPPY-2 (TTCCCCGCTACTGTCCTTC), complementary to the VP2 coding region of both BKV and JCV was used. To make the results more comprehensible, we have termed PCR products amplified by the PyV primer pair T-antigen PCR products and the products flanked by the BKTT/GPPY-2 primers NCCR PCR products.

Amplification of a β-globin gene fragment with the PCO4/GH20 primer pair was included to assess the DNA adequacy (43). PCR cycling conditions were 1 min at 94°C; then 40 cycles

* Corresponding author. Mailing address: Department of Microbiology, University Hospital of Tromsø, Box 18, N-9038 Tromsø, Norway. Phone: (+47) 776 26000. Fax: (+47) 776 27015.
of 30 s at 94°C, 10 s at 52°C, and 30 s at 72°C; and a final 3-min extension period at 72°C. Samples generating a visible fragment of the expected size in an ethidium bromide-stained 3% agarose gel were assessed as adequate for PCR analysis.

NCCR products were analyzed with restriction endonucleases Bau36I, PflM I, and SacI and DNA sequencing as earlier described (21, 48). Briefly, the BKV archetypal NCCR has arbitrarily been divided into four transcription factor binding regions called P (68 bp), Q (39 bp), R (63 bp), and S (63 bp) (33, 42, 48). The restriction enzymes Bau36I and PflM I have one recognition site in the BKV P and Q blocks, respectively, and no digestion sites in the JCV NCCR. Archetype JCV NCCR, in contrast to the BKV NCCR, contains one SacI site in a sequence domain duplicated in most progressive multifocal leukoencephalopathy-derived JCV DNAs (55).

Alkaline phosphatase-labelled oligonucleotides (British Biotechnology Products Ltd., London, England), BK and JC probes (5), were used for the detection and identification of T-antigen PCR products in a dot blot hybridization assay as earlier described (31, 48), with the following modifications. Twenty-five picomoles of each alkaline phosphatase-labelled oligonucleotide probe was added per ml of hybridization solution, and the mixtures were incubated for 30 min at 50°C. Filters were washed at 50°C for 5 min, twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate and then twice in 0.25× SSC-0.1% sodium dodecyl sulfate. After incubation at room temperature in 1× SSC for 5 min, the hybridized probe was detected on Cronex-4 films (Du Pont Co., Wilmington, Del.) by using the chemiluminescent detection reagent, LUMIPHOS 530 (Boehringer, Mannheim, Germany). Exposure was for 90 min to 6 h at 37°C. Samples yielding a clearly detectable signal with no signal from buffer and heterologous viral DNA were scored as positive.

Analysis of saliva specimens. Five microliters of each saliva lysate was used for each PCR. Only 55 of 70 samples (79%) gave the expected product by β-globin amplification. The β-globin negative specimens were spiked with 5 fg of BKV DNA (850 copies) immediately before amplification and screened for inhibition. Only four samples (6%) showed inhibition of PCR amplification (data not shown). Two of these four patients were taking oral amphotericin B, which might have inhibited the Taq polymerase.

Because of this relatively high proportion of β-globin negative specimens, two sensitive alternative PCR strategies were developed for detecting possible small amounts of viral DNA. For amplification of the T-antigen coding region the PCR cycling conditions were as earlier described (5). In addition, a sensitive heminested one-tube PCR reaction was developed (53). The BKTT1 and GPPY-2 primer pair (0.25 μM each) and the BKTT-2 primer (5 μM) flanking the viral control regions, were used. The PCR cycling conditions were 1 min at 94°C; then 20 cycles of 30 s at 94°C, 30 s at 68°C, and 30 s at 72°C; then 40 cycles of 30 s at 94°C, 10 s at 55°C, and 30 s at 72°C; and a final 3-min extension period at 72°C. No amplification products were obtained with either method, and both methods have a reproducible detection limit beyond 0.1 fg DNA (17 copies) (Fig. 1).

Analysis of NP specimens. The processed NP specimens stored at −70°C were thawed and spun at 1,500 × g for 10 min. The pellet was suspended in 200 μl of cell culture medium and divided into two 100-μl fractions. One fraction was stored at −70°C for virus isolation studies. The other fraction was boiled for 10 min and stored at −20°C for PCR analysis. Three microliters of each specimen was used for each PCR reaction. Cycling conditions were as earlier described (48).

Two of a total of 201 NP samples adequate for PCR analysis, NP 132 and 164 (from two children 7 and 8 months old), reproducibly gave PyV products by PCR. One of these children had an ongoing RSV infection, while for the other no common viral respiratory pathogen was detected by standard diagnostic procedures. Figure 2 shows a restriction enzyme analysis of the detected NCCR PCR products indicating two differently rearranged BKV NCCRs. DNA sequencing revealed one NCCR variant (NP164) earlier detected in one urine sample and two peripheral blood cell extracts from HIV-1-infected persons (48). The other sample (NP132) contained a viral control region anatomy not earlier described. P (68 bp)-Q (26 bp)-P (49 bp)-Q (39 bp)-S (63 bp), using the terminology established elsewhere (33, 48). Both sequencing and restriction enzyme analysis revealed homogeneous PCR products. Figure 3 shows the comparison of the two rearranged NCCRs in comparison to the proposed archetypal strain. It is noteworthy that both

![FIG. 1. Sensitivity of the PCR. Detection and identification of T-antigen PCR products by an alkaline phosphatase-labelled BKV-specific probe. Hybridization conditions were as described in the text. In column 5, the positive controls, amplified BKV TU DNA (row A, 0.1 fg [17 copies]; row B, 1.0 fg) were detected. In columns 1 to 4, all samples were negative. Exposure was for 3 h at 37°C. The same level of sensitivity was obtained for JCV Mad1 DNA used as a positive control for amplification of JCV DNA (data not shown).](http://jcm.asm.org/Downloaded from http://jcm.asm.org)

![FIG. 2. Analysis of amplified BK and JC virus DNA by diagnostic restriction enzyme digestion. The PCR products, amplified with the BKTT1 and -10 primers, were analyzed on a 3% agarose gel. The restriction enzymes SacI (lanes 2, 5, 8, and 11), Bau36I (lanes 3, 6, 9, and 12), and PflMI (lanes 4, 7, 10, and 13) were used. Lane 1, HinClI digest of φX174 as molecular weight markers. Lanes 2 to 4, BKV NP 132: 489 bp, 238 + 176 + 75 bp, and 270 + 144 + 75 bp, respectively. Lanes 5 to 7, BKV NP 164: 517 bp, 238 + 161 + 68 + 50 bp, and 388 + 129 bp, respectively. Lanes 8 to 10, BKV archetypal DNA: 477 bp, 239 + 238 bp comigrating, and 270 + 207 bp, respectively. Lanes 11 to 13, JCV archetypal DNA: 239 + 238 bp comigrating, 485 bp, and 485 bp, respectively. Additional faint bands due to partial digestion can be seen in some lanes.](http://jcm.asm.org/Downloaded from http://jcm.asm.org)
NP strains completely lacked the R block sequences present in the archetype.

An indirect immunoperoxidase staining method was applied for the detection of BKV antigens in infected-cell cultures in 24-well polystyrene plates (Nunc, Roskilde, Denmark) (15). Both NP specimens positive for BKV DNA in PCR analysis were inoculated on subconfluent Vero cells and a human endothelial cell line (HUV-EC-C, ATCC CRL 1730) in parallel with positive (BKV TU strain) and negative controls. A monoclonal antibody directed against BKV large T antigen and rabbit antiserum against purified BKV virions (8) were used as primary antibodies. Both samples scored negative 4 days postinfection in both cell culture systems (data not shown).

Conclusions. The results are summarized in Table 1. We have detected BKV DNA, but not infectious BKV, in 2 of 201 NP aspirates of children with respiratory infections. The identification of unique NCCR variants argues against contamination with laboratory viral strains and indicates BKV latency in some unidentified upper respiratory tract cell(s). A candidate harbor cell for the possible latent BKV genomes detected in the NP specimens might be a cell recruited from local lymphoid tissue during the ongoing infection. This is consistent with the earlier detection of BKV DNA, but not infectious virus, in tonsillar tissue (19).

Taken together, these findings suggest that neither BKV nor JCV is regularly associated with respiratory infections in children requiring hospitalization. Furthermore, the lack of infectious BKV and JCV in a considerable population of children below 5 years of age argues against the upper respiratory tract as an important site for viral replication during primary infections. However, we have screened a selected population of respiratory tract infections in children, overrepresented by RSV-associated infections. Our results do not exclude that BKV and JCV may be more prevalent in respiratory secretions from children with mild or subclinical respiratory infections.

To localize a site(s) for BKV or JCV persistence in the respiratory tract, whole-saliva samples were collected from both HIV-immunodeficient individuals and healthy controls. We have recently described an association between BKV viruria and HIV infection, and reactivation or excretion of BKV at other epithelial surfaces has been suggested (48). However, our negative findings with two sensitive PCR methods indicate that salivary glands and oropharyngeal epithelial cells are not target sites for BKV or JCV persistence. Both HIV-1 and cytomegalovirus have been detected by PCR in saliva specimens by the same approach (28).

BKV seroconversion has been linked to respiratory infections (18, 19, 32), and the rapid acquisition of specific antibodies during childhood is consistent with an infection disseminated from the respiratory tract (6, 37, 44, 50). Apart from these observations, little is known about the initial stages in PyV pathogenesis in humans. However, extrapolation from transmission studies carried out with animal PyVs may give some leads. For mouse PyV, it seems generally accepted that virus entry occurs by the respiratory route. A successful infection can be established with a smaller amount of PyV in the respiratory than in the alimentary tract (41), and primary viral replication has been demonstrated in the respiratory tract and associated tissue (11, 12). In contrast, K virus infection in mice seems to be transmitted by the oral route (20). Endothelial cells of jejunal capillaries appear to be initially infected. No viral antigen could be detected in cells of the nasopharynx, buccal mucosa, trachea, or bronchi, even after intranasal inoculation. Furthermore, nonimmune rhesus monkeys have become infected after intragastric inoculation of simian virus 40 (45, 46).

Consequently, the alimentary tract represents an interesting alternative port of entry to the host organism for members of the PyV genus, in accordance with their acid-resistant properties (39). As far as we know, this possibility has as yet been evaluated for human PyVs. Neither has the contribution of NCCR variation to PyV tissue and cell tropism been systematically approached.

Rearrangements in BKV and JCV NCCRs take place during passage in cell culture but have also been detected in vivo (36, 47–49, 52). Viral strains with different NCCRs may have divergent tropism for permissive cells and also aberrant potential for host cell transformation (1, 27, 57). Comparison of nucleotide sequences for BKV and JCV strains indicates that NCCR variants emerge from archetypes through partial duplications and/or deletions in transcriptional control elements. These rearrangements may provide the viral species as such with the ability to infect different target cells within the human organism (27). Indications have been given that naturally shed and transmitted BKV contains subpopulations with different NCCRs, permitting this species to adjust efficiently to the menu of transcription and replication factors offered by different host cells (47, 49).

The two BKV strains we have demonstrated in NP aspirates from children have grossly rearranged NCCRs. We are rea-
reasonably convinced that the infections were latent, although the ages of the two children, 7 and 8 months, might indicate a primary infection. The sensitivity of cell culture techniques in BKV detection have been questioned (35, 49). However, our interpretation is supported by the unsuccessful virus isolation attempts in a human endothelial cell line (HUC-EC-C) which is highly permissive for several BKV strains, including the archetypal BKV (WW) (20a). Also, gel electrophoresis as well as DNA sequencing revealed homogeneous PCR products. If we were picking up rearrangements during a primary infection, we would have expected to find several rearranged NCCRs represented by heterogeneous PCR products. Active virus multiplication is hence contradicted by two lines of evidence.

One of the NCCR variants (NP164) has been detected in a urine specimen and DNA extracts of peripheral blood mononuclear cells from two HIV-positive patients (48). Consequently, this NCCR anatomy seems to be stable and biologically significant. Given that this is the case also for the other, earlier undetected, NCCR variant, one must ask how these rearranged BKV strains arrived in the respiratory tract. These BKV NCCRs might have evolved during an earlier primary replication with an archetypal BKV strain. The problem is that the archetypic BKV and JCV have never been detected in materials other than kidney tissue and urine specimens, while viral strains with rearranged NCCRs have been found in various locations (14, 23, 27, 48, 52, 55, 56) and now also, for BKV, in the respiratory tract. A credible kidney-to-kidney transmission route is difficult to devise, and an efficient urethra-to-respiratory-tract transmission is dubious.

In conclusion, it seems more probable that we picked up latent genomes following earlier primary infections, possibly with rearranged BKV strains as part of a heterogeneous population (47, 49). This statement leaves the transmission route(s) and portal(s) of entry as open questions. As indicated, we propose that the alimentary tract should be considered in future investigations.

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