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The Role of BK Virus in Acute Respiratory Tract Disease and the Presence of BKV DNA in Tonsils

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The significance of BKV infections relative to infections by generally tested respiratory agents was investigated in children with acute respiratory disease. Paired sera from 177 children admitted to a hospital for acute respiratory disease were tested for significant rises in antibodies. Sera from seven patients showed a seroconversion to BKV and clinical signs of acute upper respiratory tract infection were exhibited by each of these patients.

BKV infections were present in 8% of the patients with upper respiratory tract disease while seroconversions to adenovirus (2%), influenza A virus (1%), parainfluenza virus (5%), RS virus (6%) and mycoplasma pneumoniae (1%) were observed in 15% of the patients with upper respiratory tract disease. BKV was isolated from the urine of one child with tonsillitis with a concomitant seroconversion to BKV.

Tonsils from children with recurrent attacks of acute respiratory disease were tested for the presence of BKV DNA by hybridization with a cloned genomic ^{32}P-labeled DNA of prototype BKV. Five of twelve tonsil DNAs showed hybridization with BKV DNA. Each tonsil showing hybridization with BKV DNA contained multiple nonintegrated copies of the BKV genome per diploid amount of host cell DNA.

Attempts to recover infective BKV by transfection of primary human embryonic cells with tonsil DNAs or by co-cultivation of tonsillar cells with primary human embryonic cells were unsuccessful.

Key words: BK virus, acute respiratory disease, human tonsils, nucleic acid hybridization

INTRODUCTION

Infection with the human papovavirus BK (BKV) is widespread [Brown et al, 1975] and antibodies to BKV usually occur in early childhood [Gardner, 1973]. An-

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tibody surveys in different populations revealed a 60–80% prevalence of BKV antibodies in adults [Portolani et al, 1974; Shah et al, 1973]. A mild respiratory illness in children has been recorded at the time of appearance of antibodies to BKV [Mäntyjärvi et al, 1973] and three cases of primary BKV infection have been reported in association with upper respiratory tract illness, febrile convulsions, and Guillain-Barré syndrome [van der Noordaa and Wertheim-van Dillen, 1977].

The primary BKV infection in childhood appears to be followed by a period of virus persistence. In normal healthy individuals BKV specific IgM antibodies persisted for more than 3 years suggesting that BKV continued to provide an antigenic stimulus [Flower et al, 1977]. Little data are available with respect to the site of persistence. DNA sequences hybridizing with BKV DNA have been observed in a variety of tissues by Pater et al, [1980] and in renal tissue of healthy normal individuals by Heritage et al [1981] and Grossi et al [1981].

Initially BKV has been isolated from the urine of a renal transplant recipient on immunosuppressive therapy [Gardner et al, 1971] and subsequent BKV recoveries have been obtained from individuals who were immunologically impaired [Howley, 1980] and from pregnant women [Coleman et al, 1980]. In these patients the presence of infective virus appeared to result from virus reactivation according to the presence of BKV antibodies prior to virus excretion.

Recently, BKV has been isolated from the urine of an immunocompetent child with tonsillitis and it showed subsequent seroconversion to BKV [Goudsmit et al, 1981]. The ubiquity of antibodies to BKV after the primary school years and the association of primary BKV infections with respiratory illnesses suggest that BKV spreads by the respiratory route.

In order to extend our observation on the appearance of hemagglutination-inhibiting antibodies to BKV during acute respirtory disease we have retrospectively investigated paired sera from 177 children with respiratory tract infections. The frequency of BKV infections among these patients was compared to the frequency of infections caused by commonly tested respiratory agents. In an attempt to detect the primary site of BKV replication or a possible site of BKV persistence, tonsils from children with recurrent attacks of acute respiratory disease were tested for the presence of BKV DNA. In addition we have attempted to recover infective BKV from these tonsils.

MATERIALS AND METHODS

Patients and Sera

Paired serum samples collected within a 3-year period from 177 children admitted to a hospital with acute respiratory disease were provided by the Department of Clincal Virology. Of these 177 patients, 44% (78) were between 0–1 years of age, 44% (78) between 2–5 years, and 12% (21) between 6–20 years. Upper respiratory tract infections included rhinitis, rhinopharyngitis, pharyngitis, tonsillitis, and laryngitis and occurred in 49% (86) of all cases.

Tracheitis, bronchitis, bronchiolitis, bronchopneumonia, and pneumonia were diagnosed in 51% (91) of the 177 children and were considered as lower respiratory tract infections. Febrile convulsions complicated 44 of the 86 upper respiratory tract infections and 13 of the 91 lower respiratory tract infections.

Hemagglutination-Inhibition Test (HI)

The HI test was employed to test for antibodies to BKV. The low-speed supernatant, from crude lysate of human embryonic cells infected with BKV and harvested when 75% of the cells showed cytopathic effect, was used as antigen. The highest dilution of antigen giving complete hemagglutination of human type-0 erythrocytes was considered to contain one hemagglutination unit (HAU).

Sera were pretreated with seven volumes of Receptor Destroying Enzyme (Cholera filtrate, NV Philips Duphar, Holland) for 18 hours at 37°C followed by inactivation for 1 hour at 56°C. Antigen (4 HAU) and twofold serial dilution were allowed to react for 30 minutes at 37°C before adding erythrocytes. One volume of 0.5% v/v human type-0 erythrocytes in dextrose gelatin buffer was added and incubated for 18 hours at 4°C. The highest dilution which completely inhibited hemagglutination was read at the HI titer. Paired sera were tested simultaneously and in all experiments a positive (titer: 512) and negative (titer: 8) control serum were employed as reference. A titer of at least 1:16 was considered positive.

Complement Fixation Test (CF)

The complement fixation test was employed for antibodies to adenovirus, influenza A virus, influenza B virus, parainfluenza viruses 1, 2, and 3, respiratory syncytical virus, and mycoplasma pneumoniae. The CF test was performed according to standard procedures.

Isolation of High Molecular Weight DNA

High molecular weight tonsil DNAs were extracted by a modification of procedures described by Blin and Stafford [1976]. One tonsil from each patient was quickly frozen in liquid nitrogen. Half a tonsil was used for each DNA extraction and pulverized in dry ice. The frozen powder was suspended in 0.1 M NaCl/ 0.04 M EDTA (pH 8), subsequently SDS (2%) and $NaClO_4$ (1 M) were added, immediately followed by addition of 0.5 volume of chloroform-iso-amyl-alcohol (24:1), and 0.5 volume of phenol. This mixture was shaken for 60 minutes, centrifugated at 5000g for 15 minutes, and the aqueous fase was reextracted with 1 volume chloroform-iso-amyl-alcohol (24:1). The high molecular weight DNA was precipitated in ethylene-glycol-monoethyl ether, resuspended in 10 mM Tris/1 mM EDTA (pH 8), treated with RNase (10 μg/ml), and extracted with 1 volume chloroform. The DNA was precipitated again in ethylene-glycol-monoethylether, resuspended in 10 mM Tris/1 mM EDTA (pH 8), and dialyzed extensively.

Isolation and Cloning of BKV DNA

Plaque purified BKV (prototype) was propagated at low multiplicity of infection (0.0002) in cultures of primary human embryonic cells derived from 6- to 12-week-old gestation products. All cells were cultivated in Eagle's basal medium with 10% newborn calf serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 50 units/ml mycostatin). Viral DNA was isolated from infected cells according to the method of Hirt [1967] and purified further by equilibrium centrifugation in a CsCl gradient containing ethidium bromide [Radloff et al, 1967]. BKV DNA was cloned using the pBR 322/E. coli K 12 plasmid vector system under P2 physical containment in

compliance with the NIH guidelines for recombinant DNA research [Israel et al, 1979]. BKV DNA was cloned at the single Bam HI site located at 0.98 map units [Seif et al, 1979]. The cloned DNA was used for in vitro labeling by nick translation [Rigby et al, 1977].

Restriction Endonucleases

The restriction endonucleases Bam HI, Xba I and Taq I were purified as described by Crawford and Robbins [1976].

Agarose Gel Electrophoresis

Electrophoresis, blotting, and hybridization were performed essentially as described by ter Schegget et al. [1980] except for the washing procedure after hybridization. At that point, the filters were extensively washed six times during 60 minutes at 65°C with 3 × SSC and subsequently twice during 90 minutes at 65°C with 0.1 × SSC containing denatured salmon sperm DNA (50 μg/ml) and SDS (0.1%).

Co-cultivation of Tonsillar Tissue With Primary Human Embryonic Cells

Primary embryonic human cells were derived from 6- to 12-week-old gestation products. Small (1 × 1 mm) pieces of tonsillar tissue were applied to a confluent monolayer of the embryonic cells. The cells were refed twice a week with Eagle's BME containing Earle's salts and 10% calf serum.

Transfection of Primary Human Embryonic Cells

Primary human embryonic cells were transfected with Taq I digested or undigested tonsil DNAs (10 μg/dish) by employing the DEAE-dextran method [McCutchan and Pagano, 1968]. In addition, transfection of primary human embryonic cells was performed by the calcium phosphate coprecipitation technique [Graham and van der Eb, 1973] using calf thymus DNA as a carrier. Four hours after the addition of the DNA precipitate the cells were washed once with HEPES buffer and the cells were subsequently treated with glycerol (15% v/v in HEPES buffer) for 3 minutes at 37°C. Thereafter, the glycerol was replaced by Eagle's BME containing Earle's salts and 10% calf serum. The cells were refed twice a week and subcultured after 1 month.

RESULTS

Antibodies to BKV

A significant rise in hemagglutination-inhibiting antibodies to BKV was demonstrated in paired sera of seven of 177 children tested. All seven patients lacked BKV antibodies in the first serum sample. Table I shows the clinical features of these individuals with seroconversions to BKV. Clinical signs of acute upper respiratory tract infections were found in each of these seven patients. None of these children was shown to be immunocompromised.

No significant rises in antibodies to adenovirus, influenza viruses A and B, parainfluenza viruses 1, 2, and 3, respiratory syncytial virus, and mycoplasma pneumoniae were observed in six of these seven patients (Table I) as measured by the complement fixation test. In the sera of patient 1, a concomitant rise of antibodies to adenovirus was observed.

TABLE I. Antibodies to BKV in Paired Sera of Seven Patients With Acute Respiratory Diseases

Patient[a]	Age (yrs)	Sex	HI titers (day of illness)	Clinical disease
1. Ab.[b]	1	M	< 1:8(1)-1:32(14)	Rhinopharyngitis complicated by broncho-pneumonia and febrile convulsions.
2. Gl.	17	M	1:8(2)-1:128(12)	Rhinitis complicated by sinusitis maxillaris and febrile convulsions.
3. Ko.	3	M	< 1:8(2)-1:32(12)	Rhinopoharyngitis complicated by otitis media and febrile convulsions.
4. Mo.	5	M	1:8(2)-1:256(17)	Rhinitis complicated by febrile convul-sions.
5. Re.	5	F	< 1:8(3)-1:32(14)	Rhinopharyngitis complicated by bronchitis.
6. Sp.	0.5	F	1:8(2)-1:128(20)	Rhinitis and otitis media.
7. To.	2	M	1:8(3)-1:256(14)	Acute tonsillitis.

[a]The sera of these seven patients were also tested for complement fixing antibodies against the following agents: adenovirus, influenza viruses A and B, parainfluenza viruses 1, 2, and 3, respiratory syncytial virus, and mycoplasma pneumoniae.
[b]Antibodies to adenovirus appeared in the serum of this patient.

Table II shows the number of seroconversions to BK virus, adenovirus, influenza A virus, parainfluenza virus, RS virus, and mycoplasma pneumoniae. No significant rises of antibodies to influenza B virus have been detected in these 177 children. The mean age ($\bar{x} \pm$ SEM) of the children with a primary BKV infection (4.8 ± 2.2) did not differ significantly from the mean age of the children with primary infections caused by adenovirus (2.6 ± 1.2), influenza A virus (3.3 ± 0.4), parainfluenza virus (2.1 ± 0.5), RS virus (2.7 ± 0.4), or mycoplasma pneumoniae (2.1 ± 1.0). All BKV infections were associated with upper respiratory tract disease while in the majority of the infections caused by the other investigated agents a lower respiratory tract illness was observed (Table II).

Febrile convulsions occurred in 4 of 7 BKV infections and in 9 of 13 upper respiratory tract infections by adenovirus, influenza A virus, parainfluenza virus, RS virus, and Mycoplasma pneumoniae. Antibodies to BKV appeared in 7 (8%) of all 86 patients with upper respiratory tract infections. Seroconversions to adenovirus (2%), influenza A virus (1%), parainfluenza virus (5%), RS virus (6%), and mycoplasma pneumoniae (1%) were observed in 15% of all 86 patients with upper respiratory tract disease, respectively.

We were able to isolate a papova virus from the urine of one patient (patient 7, Table I). Biological and immunologic characterization indicated that the virus was a variant of the human papova virus BK (BKV) [Goudsmit et al, 1981].

Hybridization of BKV DNA

Five of twelve tonsil DNAs showed hybridization with BKV DNA after digestion with Bam HI, an enzyme which cleaves BKV DNA once at 0.98 map units (Fig. 1). These bands comigrated with linear BKV DNA indicating the presence of genome-length nonintegrated BKV DNA. In order to confirm these results high molecular weight DNAs were isolated from another part of tonsils 4, 5, and 6 and digested with Taq I, Bam HI, and Xba I (Fig. 2).

TABLE II. Seroconversions in Paired Sera From 177 Hospitalized Children With Acute Respiratory Disease

Disease	Number of cases with seroconversions against: BKV[a]	AD	IA	Pa	RSV	Mp
Upper respiratory tract disease (n = 86)	7	2	1	4	5	1
Lower respiratory tract disease (n = 91)	–	3	2	4	10	3
Totals (n = 177)	7	5	3	8	15	4

[a]BKV = BK virus, Ad = adenovirus, IA = influenza A virus, Pa = parainfluenzavirus, RSV = respiratory syncytial virus, Mp = Mycoplasma pneumoniae.

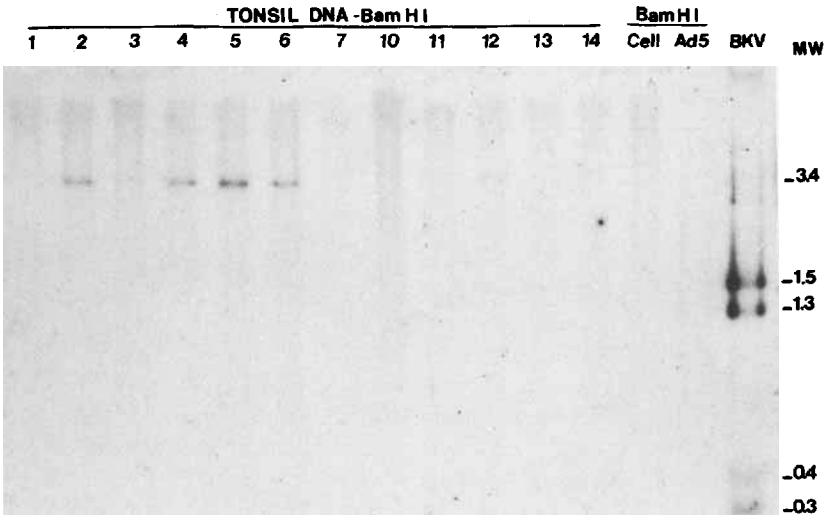

Fig. 1. Hybridization of cloned genomic ^{32}P-labeled DNA of BKV(prototype) with 12 tonsil DNAs after cleavage with the restriction endonuclease Bam HI. Five of the 12 tonsils (tonsils 2,4,5,6, and 12) showed hybridization with BKV DNA. Tonsil DNAs (10 μg) were digested with Bam HI for 2 hours at 37°C in the appropriate buffer. Forty copy equivalents of Hin d III digested BKV DNA, 100 copy equivalents of Bam HI digested adenovirus type 5 DNA, Bam HI digested cellular DNA (10 μg), and 12 tonsil DNAs (10 μg) were electrophoresed on 1% agarose gels, transferred to nitrocellulose filters, annealed with ^{32}P-labeled BKV DNA (complete genome; specific activity:2×10^8 cpm/μg DNA), and autoradiographed. Molecular weights ($\times 10^6$ daltons) are indicated.

Digestion of these tonsil DNAs with Taq I showed predominantly one band comigrating with Form III DNA of prototype BKV (Fig. 2). Cleavage of tonsil 6 DNA with Taq I revealed two additional restriction fragments of 2900 and 1500 base pairs. Cleavage of tonsils 4, 5, and 6 with the single cut enzyme Bam HI revealed one predominant band comigrating with Form III DNA of BKV. BKV DNA sequences were further analyzed by cleavage of tonsil DNA with Xba I. Xba I which cleaves BKV DNA twice at 0.23 and 0.96 map units yielded predominantly two restriction fragments comigrating with the Xba I restriction fragments of prototype BKV DNA (Fig. 2).

No BKV was isolated either by co-cultivation of tonsillar tissue with primary human embryonic cells or after transfection of primary human embryonic cells with

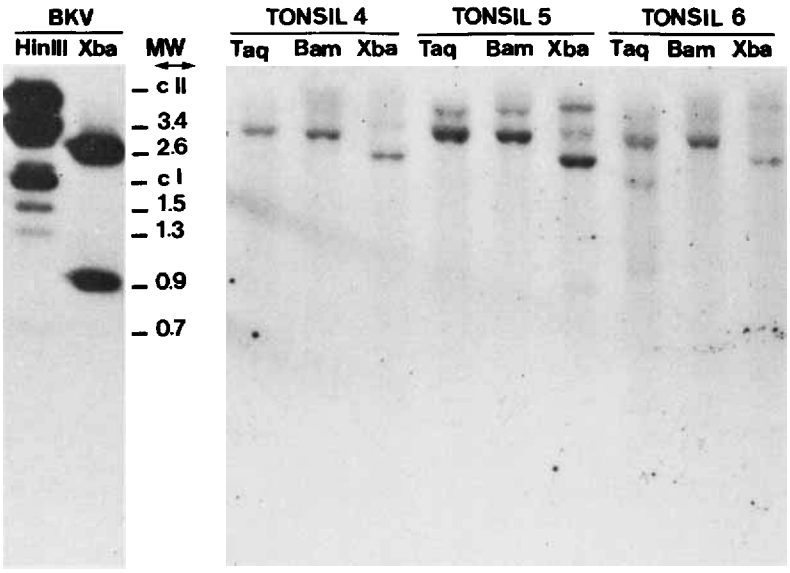

Fig. 2. Hybridization of cloned genomic ^{32}P-labeled DNA of BKV (prototype) with tonsil DNAs 4, 5, and 6 after cleavage with the restriction endonucleases Taq I, Bam HI, and Xba I. Tonsil DNAs (10 μg) were digested with Taq I, Bam HI, and Xba I for 2 hours at 65°C (Taq I) or 37°C (Bam HI, Xba I) in the appropriate buffers. BKV DNA was digested with Hin d III (40 copy equivalents) and with Xba I (20 copy equivalents) for 2 hours at 37°C in the appropriate buffers. All DNAs were electrophoresed on 1.5% agarose gels, transferred to nitrocellulose filters, annealed with ^{32}P-labeled BKV DNA (complete genome; specific activity: 1.5×10^8 cpm/μg DNA) and autoradiographed. Molecular weights ($\times 10^6$ daltons) are indicated. C I and C II reflect the supercoiled and relaxed molecules of BKV DNA. Both panels are from the same gel and arrows indicate the top of the gel.

tonsillar DNA. Sera were obtained from two children whose tonsils contained BKV DNA (tonsils 2 and 6). Hemagglutination-inhibiting antibodies to BKV were present in both sera. Sera were also obtained from six children whose tonsils did not contain BKV DNA (tonsils 1, 7, 10, 11, 13, and 14). Hemagglutination-inhibiting antibodies to BKV were present in five (tonsils 1, 7, 10, 13, and 14) of the six sera.

DISCUSSION

Paired sera from seven children with acute respiratory disease showed a seroconversion to BKV in the course of their illness, and in one case of tonsillitis BKV was isolated as well. None of these children appeared to be immunologically impaired. In our study, BKV had a distinct preference for the upper respiratory tract, considering that acute infections of the upper and lower respiratory tract were almost equally distributed among the 177 children tested.

Acute infections by adenovirus, influenza A virus, parainfluenza virus, RS virus, or Mycoplasma pneumoniae were more often associated with lower respiratory tract diseases (Table II). The prevalence of BKV antibodies in children with acute respiratory disease did not differ significantly from the BKV antibody prevalence in the normal population at various ages (data not shown). This can be explained by the high preva-

lence of BKV antibodies in the normal population and the frequent occurrence of subclinical infections.

Subsequent analysis of DNAs from tonsils of children suffering from recurrent attacks of acute respiratory disease revealed the presence of BKV DNA in five of 12 tonsils tested. Digestion of tonsil DNAs with the restriction endonucleases Bam HI and Xba I indicated that multiple free copies of the BKV genome per diploid amount of host cell DNA were present in each tonsil showing hybridization with BKV DNA. Neither co-cultivation of tonsillar tissue with permissive cells nor transfection of permissive cells with tonsil DNAs yielded infectious BKV even though multiple nonintegrated BKV DNA copies per cell could be detected. Recently, Heritage et al [1981] and Grossi et al [1981] reported the presence of BKV DNA sequences in kidneys of aged people. Explant cultures set up from one cadaver kidney containing BKV DNA sequences remained negative for BKV and BKV antigens [Heritage et al, 1981].

The inability to recover infectious virus from these tonsils or to demonstrate the infectivity of the BKV sequences in tonsillar tissue suggest that the episomal BKV DNAs in tonsils of young children represent some sort of quiescent state of the viral genome. The presence of BKV-specific antibodies in sera from children whose tonsils did not contain BKV DNA sequences indicates that BKV infections also occur without subsequent detectable levels of BKV DNA sequences in tonsillar tissue. The observed seroconversions to BKV in seven cases of acute upper respiratory tract disease, the isolation of BKV from the urine of one of those cases, and the occurrence of BKV DNA sequences in tonsillar tissue of young children strongly suggest that BKV spreads in early childhood by the respiratory route.

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