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Detection of polyomavirus SV40 in tonsils from immunocompetent children

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Abstract

Background—BK virus (BKV), JC virus (JCV) and simian virus 40 (SV40) are nonenveloped DNA viruses, members of the family *Polyomaviridae*. BK and JC viruses establish persistent infections in humans, and evidence suggests that SV40 can infect humans, as well. Whether persistence occurs in the lymphoid system is unknown.

Methods—Paraffin-embedded tonsils from 220 immunocompetent children (mean age 9.3 years) were examined by polymerase chain reaction (PCR) to detect viral DNA of BKV, JCV, SV40, and Epstein-Barr virus (EBV).

Results—Polyomavirus-specific DNA sequences were detected in 8.3% (29/351) of specimens collected from 220 children. Twenty-one (9.5%) children had polyomavirus DNA present in at least one tonsil, with sequences identified as SV40 (n=20) and BKV (n=1). Polyomavirus JCV was not detected. Among patients positive for SV40, 8 of 14 (57%) contained viral DNA in both available tonsils. EBV DNA was detected in 99 (28.2%) samples from 67 (30.5%) patients. Eleven samples (3.1%) from 8 (3.6%) children were positive for both polyomavirus and EBV. SV40-positive children were significantly older than the SV40-negative subjects ($P < 0.001$). T-antigen expression was detected in an SV40 DNA-positive tonsil by immunohistochemistry.

Conclusions—These results suggest that SV40 can infect tonsils, that lymphoid tissue may represent a site for polyomavirus persistence, and that immunohistochemistry is not a useful detection assay when there are very few virus-infected cells in a tissue.

Keywords

Polyomavirus; BK virus; SV40; EB virus; Tonsils; Lymphoid tissue; Persistence

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1. Introduction

Polyomaviruses BK virus (BKV) and JC virus (JCV) persist in humans following infection early in life. Approximately 60–80% of the human population produce antibodies to BKV and JCV by the second decade of life, with seroconversion occurring mostly during childhood and earlier for BKV than for JCV (Imperiale and Major, 2007; Knowles, 2006; Major et al., 1992; Walker and Frisque, 1986). Simian virus 40 (SV40), a polyomavirus of macaque origin, was introduced into humans presumably as a result of the widespread use of poliovaccines inadvertently contaminated with this virus (Butel and Lednicky, 1999; Cutrone et al., 2005; Stratton et al., 2003; Vilchez and Butel, 2004). The contamination occurred because vaccines were produced in cultures of kidney cells derived from rhesus macaques, which are frequently infected with SV40. Millions of people worldwide were exposed to live SV40 via these early vaccines (Butel and Lednicky, 1999; Cutrone et al., 2005; Proceedings of the Second International Conference on Live Poliovirus Vaccines, 1960; Stratton et al., 2003; Vilchez and Butel, 2004). The Institute of Medicine concluded that some SV40 infections in the human population today are related to SV40 exposure from early forms of the poliovaccine (Stratton et al., 2003). SV40 has been detected in specimens from children and adults not exposed to known contaminated poliovaccines, suggesting that SV40 is transmitted in humans today (Butel and Lednicky, 1999; Stratton et al., 2003; Vilchez and Butel, 2004).

BKV, JCV, and SV40 have been detected in a variety of organ systems including urinary, central nervous, and gastrointestinal (Khalili and Stoner, 2001; Major et al., 1992; Vanchiere et al., 2005a, 2005b). This suggests that the pathogenesis of these viruses involves dissemination within the host. While the route of viral spread is unknown, BKV, JCV, and SV40 DNA sequences have been identified in B-lymphocytes from healthy blood donors and immunosuppressed patients (Azzi et al., 1996; Chatterjee et al., 2000; David et al., 2001; Dolei et al., 2000; Martini et al., 1996; Monaco et al., 1996). Both BKV and JCV DNA have been found in tonsil tissue in children, and JCV DNA has been found in spleen and lymph nodes (Dorries et al., 1994; Goudsmit et al., 1982; Kato et al., 2004; Monaco et al., 1998). These findings suggest that the lymphoid system plays a role in polyomavirus infections and may be a site for polyomavirus persistence. Epstein-Barr virus (EBV) is a herpesvirus found in a majority of the human population. After primary infection the virus maintains a state of lifelong, asymptomatic latent persistence in lymphoid tissues with sporadic reactivation (Rickinson and Kieff, 2007).

SV40, like other polyomaviruses, is known to establish persistent infections. The level of virus present may be very low in immunocompetent hosts (Butel, 2000; Lednicky and Butel, 1999), although SV40 can cause widespread infections in immunocompromised monkeys (Lednicky et al., 1998). The nature of interaction of SV40 with lymphoid tissue is unclear. B-cell lymphomas occur in small-animal models of SV40 infection (Cicala et al., 1992; Diamandopoulos, 1973; Matthews et al., 1987), and SV40 is associated with some B-cell lymphomas in humans (Amara et al., 2007; David et al., 2001; Meneses et al., 2005; Shivapurkar et al., 2002; Vilchez et al., 2002b; Zekri et al., 2007). Therefore, B-cell-rich tissues such as tonsils may represent a site that harbors SV40.

The objective of this study was to test the hypothesis that polyomaviruses establish infections in lymphoid tissue. We investigated the frequency of infection by polyomaviruses in tonsils of immunocompetent children. The results suggest that tonsillar tissue is a site for SV40 infection and might play a role in viral persistence.

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2. Patients, Materials, and Methods

2.1. Patients and lymphoid tissue specimens

A total of 385 paraffin-embedded, nonmalignant tonsils from 237 immunocompetent children from November 2003 to June 2006 were obtained from Texas Children's Hospital, Houston, Texas. Demographic data collected on patients included age, gender, and pathologic diagnosis. The study was approved by the Institutional Review Board at Baylor College of Medicine. The most common reason for removal of tonsils from children at this hospital is tonsillar hypertrophy. Currently, only gross examination is performed on all tonsils. Previously, when tonsils were examined microscopically, the majority were designated as having "benign lymphoid hyperplasia". That was the case for both virus-positive and virus-negative tonsils in this study for which microscopic evaluations were available. A retrospective chart review of 19 of the SV40-positive patients revealed that all had a diagnosis of tonsillar hypertrophy.

2.2. DNA extraction from tonsil samples

Paraffin-embedded tonsillar tissues (two 20-μm sections) were deparaffinized with xylene and then washed with 100%, followed by 70%, ethanol. DNA was extracted using a proteinase K and phenol/chloroform extraction method as previously described (Lednicky and Butel, 1998). All sample processing was performed in a laminar flow hood within a BSL-2 facility free from viruses and plasmids.

2.3. PCR amplification and DNA sequence analysis

Polymerase chain reaction (PCR) assays were set-up in a "PCR Clean Room" using barrier pipette tips to avoid contamination. For each sample, a total of 250–500 ng of DNA was used per reaction. All DNA samples were tested for suitability for amplification using primers specific for the human β-hemoglobin gene (primers P0C3/KM38) (Vilchez et al., 2002b) and only specimens from which cellular sequences were amplified (n=351) were examined further. Primers PYVfor/PYVrev directed at a conserved sequence in the amino (N)-terminus of the large T-antigen (T-ag) gene of BKV, JCV, and SV40 were used for viral analysis (Bergsagel et al., 1992; Lednicky et al., 1995). Our laboratory has established the use of these primers to detect BKV and JCV in clinical samples (Thomas et al., 2007; Vanchiere et al., 2005a, 2005b) as well as SV40 (Meneses et al., 2005; Thomas et al., 2007; Vilchez et al., 2002b). Primers TP1Q3/TP1Q5 were used to detect EBV latent membrane protein 2a (LMP2a) gene sequences (Vilchez et al., 2002a). Positive control plasmids were added to the control PCR reactions outside of the clean rooms after tubes containing negative controls and test DNAs were closed. The positive controls were a plasmid containing cloned SV40 (pSV40-B2E) (McNees et al., 2005) for polyomavirus PCR reactions and a plasmid containing cloned LMP2a gene (O'Sullivan et al., 2002) or cellular DNA from the EBV-positive lymphoma cell line Namalwa (Meneses et al., 2005) for EBV PCR reactions. Negative controls for PCR assays were reactions without added DNA template. Two to 10 separate PCR reactions were run per sample for the polyomavirus assay and two PCR reactions for the EBV assay. PCR products were sequenced to confirm the identity of polyomavirus DNA recovered from lymphoid tissues and only sequence-proven cases are reported here. Samples for which sequence analysis of a PCR product was not successful were not designated as virus-positive tissues.

2.4. Immunohistochemistry

Immunohistochemical staining for detection of SV40 T-ag was as described (Meneses et al., 2005), except that citrate buffer (pH 6) was used for antigen retrieval and the monoclonal antibody against SV40 T-ag (PAb101) was used at a dilution of 1:5000. SV40-induced hamster tumors served as positive controls.

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2.5. Statistical analysis

Differences among samples were calculated with the 2-tailed Student's *t* test using statistical software (STATA 9.0 for Windows). Differences among groups were considered statistically significant at $P < 0.05$.

3. Results

3.1. Detection of SV40 and EBV DNA sequences in lymphoid tissue samples

DNAs were extracted from 385 tonsil specimens from 237 healthy children (Table 1). Some specimens represented separate left and right tonsils from the same patient. DNAs were screened for suitability for PCR analysis by amplifying a cellular β-globin gene sequence. β-globin gene sequences were successfully amplified from 351 (91%) of 385 lymphoid specimens from 220 (93%) of 237 subjects. These included both left and right tonsils from 131 patients. These specimens were analyzed for the presence of polyomavirus and EBV DNA sequences.

Polyomavirus DNA was detected in 29 (8.3%) of 351 tonsil samples from 21 (9.5%) of 220 immunocompetent children (Table 1). Sequence analysis of the polyomavirus large T-ag PCR amplicons revealed that 28 contained sequences unique to SV40, whereas BKV DNA sequence was present in one sample. JCV DNA sequences were not detected in tissue samples. Among the 20 SV40-positive patients, samples of both left and right tonsils were available from 14 patients, 8 (57%) of whom contained detectable SV40 DNA in both tonsils. EBV LMP2a gene DNA was detected in 99 (28.2%) of 351 tonsil samples from 67 (30.5%) of 220 children (Table 1). Among the 67 EBV-positive patients, 28 of 48 (57%) contained EBV DNA in both left and right tonsils. Eleven (3.1%) samples from 8 (3.6%) children were positive for both polyomavirus and EBV DNAs.

Specimens in which polyomavirus DNA was present were tested for T-ag expression by immunohistochemistry (IHC). T-ag gene expression was detected in 1 (4.8%) of 21 tissues tested, a sample that was SV40 DNA-positive (Fig. 1).

3.2. Clinical Correlations

The demographics of the study population are shown in Table 2. The mean age of the study population was 9.3 years, ranging from 2 years to 18 years. The mean age of children whose tonsils were SV40-positive was 12.3 years (range 10–17 years) compared to 8.7 years (range 2–18 years, $P < 0.001$) for children whose tonsils were SV40-negative (Fig. 2). The BKV-positive patient was a 3-year-old male. The mean age of children who were EBV DNA-positive was 9.7 years (range 3–18 years), compared to 9.1 years (range 2–17 years, $P = 0.29$) for children whose tonsils contained no detectable EBV DNA (Fig. 2).

Of the polyomavirus-positive patients, 33% were Caucasian, 33% were Hispanic, 19% were African-American, 5% were Asian-American, and 10% were other or not specified. This is similar to the ethnicities of the Texas Children's Hospital patient population of 38,579 patients from the same time period (34% Caucasian, 31% Hispanic, 19% African-American, 2% Asian-American, and 14% other or unknown).

4. Discussion

This investigation demonstrates that polyomaviruses SV40 and BKV can be present in tonsils from immunocompetent children. Polyomavirus DNA was detected in tonsils from 21 (9.5%) children, with SV40 detected most frequently [20/21 (95.2%)]. To our knowledge, this is the first report of the presence of SV40 DNA in human tonsillar tissue. The validity of these

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findings is substantiated by the fact SV40 was detected in both left and right tonsils in over half of the SV40-positive children from whom both tonsils were available. The mean age of children with SV40-infected tonsils was significantly older than children in whom polyomavirus DNA was absent. EBV DNA was also found in tonsils from this patient population (30.5%), with no age difference between the EBV-positive and -negative children. Previous reports have estimated the prevalence of EBV in nonneoplastic tonsils to be between 29% and 51% (Endo et al., 2001; Pai et al., 2004) and our findings were similar (30%). Eight patients (3.6%) had tonsillar coinfections with SV40 and EBV.

These findings support the hypothesis that polyomaviruses infect the human lymphoid system. The frequency of detection of BKV and JCV in the present study is lower than reported for the few studies in the published literature (Goudsmit et al., 1982; Kato et al., 2004; Monaco et al., 1998). These results probably reflect differences in samples and detection methods used. Goudsmit et al. (1982) utilized hybridization of ^{32}P-labeled BKV DNA to tonsil DNAs that had been cleaved with restriction enzyme BamHI. Half a frozen tonsil was used for each DNA extraction and a large amount (10 μg) of tonsil DNA was added per lane for gel electrophoresis. Five of 12 tonsils from young children yielded a DNA band of the correct size that hybridized to BKV DNA. Monaco et al. (1998) tested tonsils from children (3–15 yr) and adults (18–57 yr) for JCV using a nested PCR approach followed by Southern blot hybridization. One microgram of tonsil DNA was used per reaction. Using this sensitive approach, 19/54 (35%) tonsils were JCV-positive. Kato et al. (2004) tested tonsillar tissues from adults (21–61 yr) for JCV, also using a nested PCR assay. Ten reactions with 1–2 μg DNA/reaction were run per sample to increase sensitivity to detect very low copy numbers of viral DNA. JCV DNA was detected in 14 of 32 donors (44%). For 10/17 (59%) JCV-positive tonsils, only 1 of 10 replicate nested PCR reactions was positive. In contrast to these studies, we extracted DNA from only two 20-micron sections from each formalin-fixed, paraffin-embedded tonsil and used 250–500 ng of DNA per PCR reaction. We did not use a nested PCR assay and in those cases in which we were able to run replicate PCR reactions, similar to the observation of Kato et al. (2004), usually a single reaction was positive for polyomavirus DNA. This suggests a very low viral load in the tonsillar tissue, with SV40 perhaps tending to be higher than BKV in this patient population. Considering that T-ag expression was detected in a single tonsil, this shows that IHC is not a useful screening test when there are very few virus-infected cells in a tissue. The absence of JCV in this study probably reflects the young age of our study population (mean 9.3 years) as JCV infections tend to occur in older children and adolescents (Kato et al., 2004; Knowles, 2006; Monaco et al., 1998). The detection of SV40 DNA in tonsil tissue (9.1%) in this study was similar to the seroprevalence of SV40 in hospitalized children (5.9%) in the Houston area (Butel et al., 1999). In addition, a recent study of stool samples from 99 children at the same hospital found that 8% and 38% of children excreted polyomavirus SV40 and BKV, respectively (Vanchiere et al., 2005a).

Sources of transmission for BKV and JCV are thought to be urinary, gastrointestinal, and/or respiratory tracts (Knowles, 2006; Ling et al., 2003; Vanchiere et al., 2005a), but transmission of SV40 remains unknown. Results described here suggest that tonsillar tissue represents a site of infection by SV40, but whether the virus reached the tonsils via blood or an oral or respiratory route is not known.

The presence of SV40 in tonsils adds to indications that SV40 can infect B-cells. SV40 DNA sequences have been detected in B-lymphocytes from healthy blood donors and immunosuppressed patients (David et al., 2001; Dolei et al., 2000; Martini et al., 1996). B-cell lymphomas arise in some hamsters inoculated with SV40 (Cicala et al., 1992; Diamandopoulos, 1973; Matthews et al., 1987) and human B-cell lymphomas were found in recent investigations to contain SV40 markers (Amara et al., 2007; David et al., 2001; Meneses et al., 2005; Nakatsuka et al., 2003; Shivapurkar et al., 2002; Vilchez et al., 2002b; Zekri et

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al., 2007). Our findings here suggest that B-cell-rich tonsil tissue might play a role in viral persistence.

In summary, the detection of polyomavirus DNA in tonsils from pediatric patients supports the hypothesis that these viruses can infect lymphoid tissue. Further studies are needed to delineate the significance of polyomavirus infections in lymphoid tissue and the possibility of tonsillar tissue serving as a reservoir for polyomavirus persistence.

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Abbreviations

BKV
: BK virus

JCV
: JC virus

SV40
: simian virus 40

EBV
: Epstein-Barr virus

PCR
: polymerase chain reaction

T-ag
: large tumor antigen

IHC
: immunohistochemistry

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Zekri AR, Mohamed W, Bahnassy A, Refat L, Khaled M, Shalaby S, Hafez M. Detection of simian virus 40 DNA sequences in Egyptian patients with different hematological malignancies. Leuk Lymphoma 2007;48:1828–34. [PubMed: 17786720]

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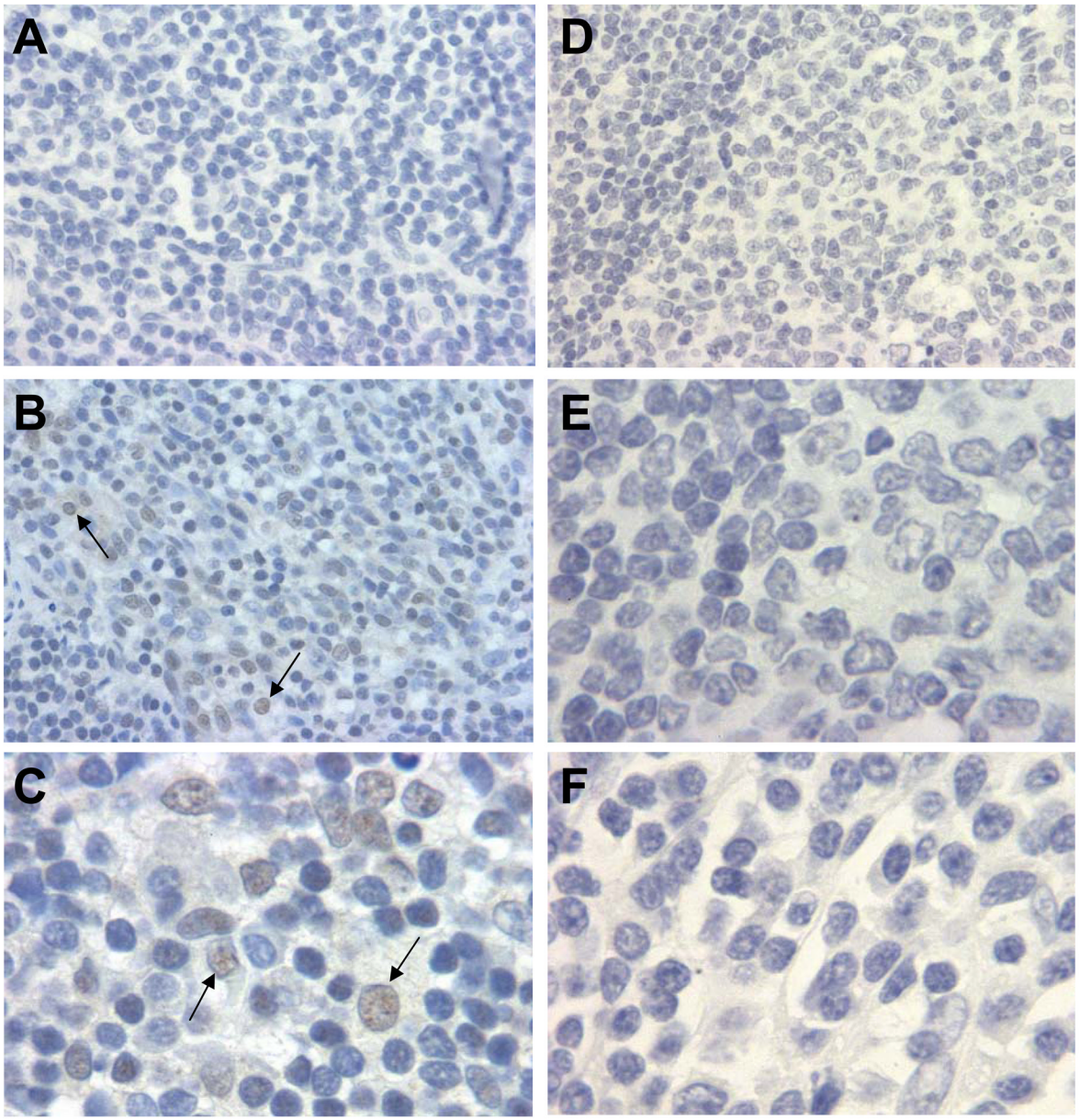

Fig. 1.

(A–C) SV40 T-ag expression in an SV40 DNA-positive tonsil from a 14-year-old male. (A) No staining with negative mouse antibody control. (B,C) SV40 T-ag-positive staining with antibody PAb101. (D–F) Negative T-ag staining with antibody PAb101 on other tonsils (D and E are the same specimen). Arrows point to representative T-ag-positive cells in panels B and C. Original magnification for panels A, B and D, 40×; panels C, E and F, 100×.

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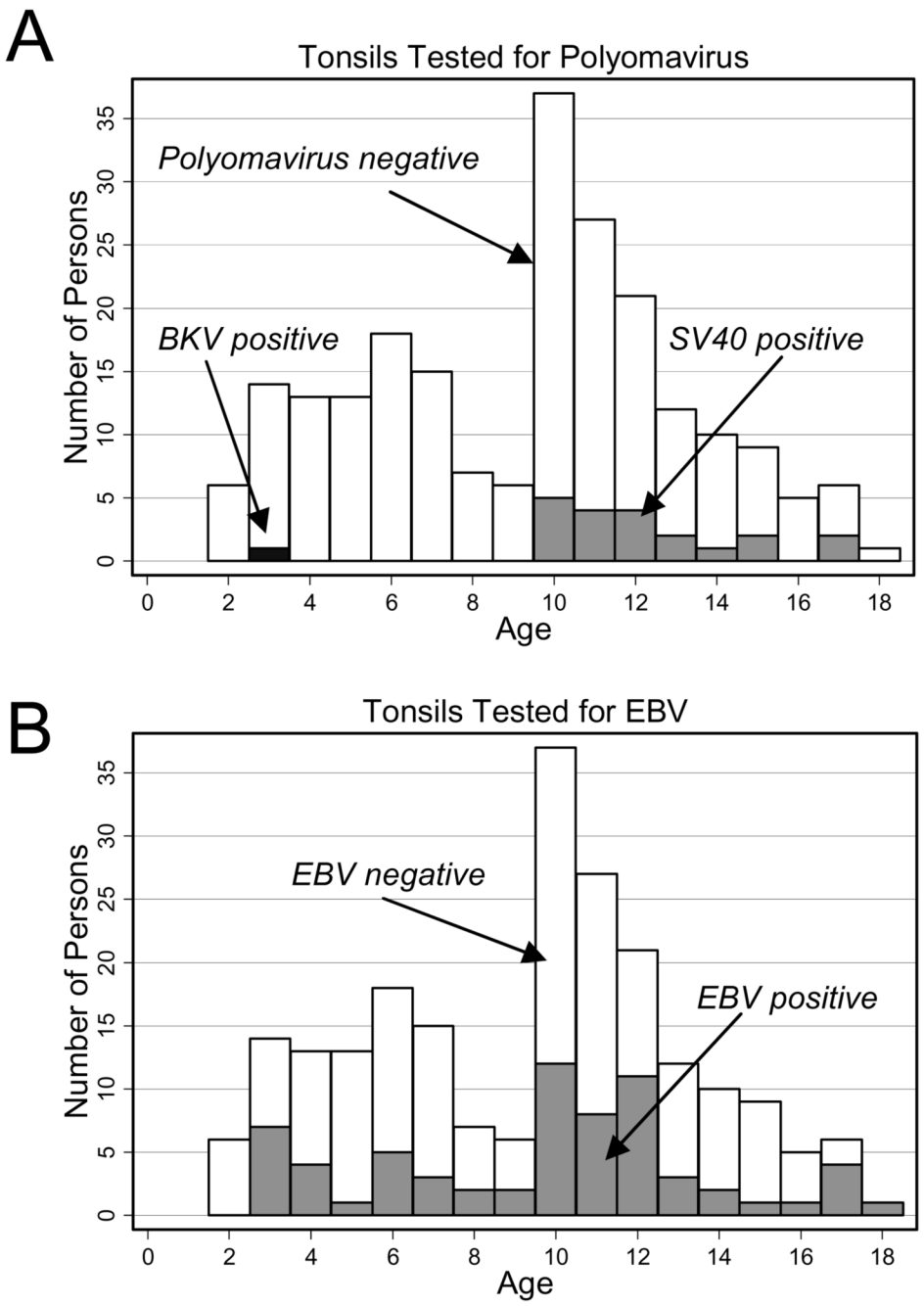

Fig. 2.

Age distributions among children with virus-positive tonsils. (A) Histogram plot of the age distribution of children with SV40-positive (grey bar) and SV40-negative (white bar) tonsils. SV40-positive children were significantly older (12.3 yr vs. 8.7 yr, respectively; $P < 0.001$). One tonsil from a 3-year-old male was positive for BKV DNA (black bar). (B) Histogram plot of the age distribution of children with EBV-positive (grey bar) and EBV-negative (white bar) tonsils (9.7 yr vs. 9.1 yr, respectively; $P = 0.29$).

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Table 1

Presence of polyomavirus and Epstein-Barr virus sequences in tonsils from immunocompetent children[a]

No. of patients	No. DNA-positive patients (%)[b]				No. of tissues	No. of DNA-positive tissues (%)[b]			
	β-globin	PYV T-ag[c]	EBV LMP2a	Both PYV and EBV		β-globin	PYV T-ag	EBV LMP2a	Both PYV and EBV
237	220 (92.8)	21 (9.5)[d]	67 (30.5)	8 (3.6)	385	351 (91.2)	29 (8.3)	99 (28.2)	11 (3.1)

[a] Polymerase chain reaction (PCR) assay was used to detect DNA sequences from paraffin-embedded tonsil tissue. Human β-globin gene, a cellular-control gene, was analyzed by PCR first to determine suitability of DNA. Only those samples that amplified the human β-globin gene were analyzed further. Polyomavirus PCR products were DNA sequenced to confirm the presence and identity of the polyomavirus type present.

[b] The numbers of patients (n=220) and tissues (n=351) that yielded a positive reaction for β-globin DNA were used as baselines to calculate the percent viral DNA-positive values.

[c] Abbreviations used: PYV, polyomavirus; T-ag, large tumor antigen; EBV, Epstein-Barr virus; LMP2a, latent membrane protein 2a.

[d] Of polyomavirus-positive tonsils from 21 children, 20 contained simian virus 40 and 1 contained BK virus-specific DNA sequences.

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Table 2

Demographic characteristics of study population of immunocompetent children[a]

Characteristics	Total	SV40 in tonsils		*P* value	EBV in tonsils		*P* value
		SV40 positive	SV40 negative		EBV positive	EBV negative	
Age (years)							
Mean age	9.3	12.3	8.7	<0.001	9.7	9.1	0.29
Age range	2–18	10–17	2–18		3–18	2–17	
Female/male	116/104	7/13	109/91		32/35	82/71	
Total subjects	220	20	200		67	153	

[a]Abbreviations used: SV40, simian virus 40; EBV, Epstein-Barr virus.