The Dynamics of Herpesvirus and Polyomavirus Reactivation and Shedding in Healthy Adults: A 14-Month Longitudinal Study

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Humans are infected with viruses that establish long-term persistent infections. To address whether immunocompetent individuals control virus reactivation globally or independently and to identify patterns of sporadic reactivation, we monitored herpesviruses and polyomaviruses in 30 adults, over 14 months. Epstein-Barr virus (EBV) DNA was quantitated in saliva and peripheral blood mononuclear cells (PBMCs), cytomegalovirus (CMV) was assayed in urine, and JC virus (JCV) and BK virus (BKV) DNAs were assayed in urine and PBMCs. All individuals shed EBV in saliva, whereas 67% had ≥1 blood sample positive for EBV. Levels of EBV varied widely. CMV shedding occurred infrequently but occurred more commonly in younger individuals (P < .03). JCV and BKV virurias were 46.7% and 0%, respectively. JCV shedding was age dependent and occurred commonly in individuals ≥40 years old (P < .03). Seasonal variation was observed in shedding of EBV and JCV, but there was no correlation among shedding of EBV, CMV, and JCV (P > .50). Thus, adults independently control persistent viruses, which display discordant, sporadic reactivations.

Herpesviruses and polyomaviruses are known to establish latent and/or persistent infections in humans, to undergo periodic reactivation and replication, and to cause disease in susceptible hosts. The majority of the studies of infections by these viruses have focused on acute diseases in individuals with congenital, acquired, and/or iatrogenic immunosuppression [1–4]. Little is known of the comparative patterns of reactivations displayed over time by immunocompetent individuals.

Epstein-Barr virus (EBV), a herpesvirus, is estimated to infect >90% of the human population. After primary infection, a life-long carrier state develops in which infectious virus may be detected in both saliva and latently infected peripheral B–lymphocytes [3, 5, 6]. EBV is associated with several human cancers, including Burkitt lymphoma, lymphoproliferative disorders in organ-transplant and bone marrow–transplant recipients, and nasopharyngeal carcinoma [3, 5, 7]. Cytomegalovirus (CMV), another herpesvirus, is estimated to infect ~50% of the population outside urban areas and ~90% inside urban areas. In healthy individuals, the most common and least serious type of CMV disease is a mononucleosis-like syndrome [1, 2]. In contrast, almost all seropositive organ-transplant and bone marrow–transplant
patients experience reactivation of latent CMV, and most seronegative recipients develop primary infections. Interstitial pneumonia is considered the most serious complication of CMV infection in transplant patients, and CMV is the most frequently identified cause in fatal cases [1, 2]. With both EBV and CMV, high levels of stress might induce transient immunosuppression and might perturb normal immune responses that contain viral reactivation, which might result in increased levels of viral shedding [8–11].

JC virus (JCV) and BK virus (BKV) are polyomaviruses that cause primary infections at an early age and that appear to be mostly asymptomatic. These viruses are ubiquitous and infect ≳70% of adults [4]. JCV and BKV establish latent/persistent infections in the kidneys and may be shed in the urine [12], but there are conflicting reports on their frequencies in the blood of healthy individuals [13–16]. Progressive multifocal leukoencephalopathy is the best-known clinical syndrome caused by JCV [4]. In patients with hematological malignancies and congenital immunodeficiency syndromes, BKV causes disease of the genitourinary tract. However, the group that is most commonly affected by BKV is transplant patients, including kidney and bone-marrow recipients [4]. JCV and BKV have the ability to induce tumors in laboratory animals, and they have been associated with some human solid cancers [17, 18].

With these factors in mind, we sought to determine the patterns of reactivation and shedding, over time, of these viruses, which are known to cause persistent infections in humans. We focused on herpesviruses EBV and CMV and on polyomaviruses JCV and BKV, because of their high prevalences in humans and the increased recognition of clinical syndromes related to their reactivations. In addition, these pathogens target different organs in the host and can be detected in easily obtainable specimens. We hypothesized that host factors control different persistent infections independently and that, over time, this would be reflected, among virus types, in sporadic, discordant viral shedding.

PATIENTS, MATERIALS, AND METHODS

Study group. Thirty immunocompetent adult volunteers from Houston, Texas, were enrolled prospectively. Inclusion criteria were the following: age >18 years and no history of diseases associated with congenital or acquired immune deficiency or of treatment with antiviral or immunosuppressive drugs, such as steroids. Women who were pregnant were excluded. Blood, saliva, and urine samples were collected at 2-month intervals from November 1998 through December 1999. Subjects were screened, by use of questionnaires, for symptoms of acute illnesses—such as fever, cough, sore throat, body aches, and diarrhea—at each collection time. Individuals were defined as low viral shedders if they had shed a given virus on 0–1 occasions, as moderate shedders if they did so on 2–3 occasions, and as high shedders if they did so on >3 occasions, over the 14-month study period. An individual who was known to shed JCV in urine donated urine specimens, which served as internal controls for JCV-detection methods.

Processing of blood, saliva, and urine specimens. Fifty milliliters of whole blood was obtained by peripheral venipunctures, by antiseptic techniques, into acid-citrate-dextrose collection tubes, and the peripheral blood mononuclear cells (PBMCs) were selectively collected after differential centrifugation through a ficoll-hypaque gradient (Accuspin system-Histopaque-1077 centrifuge tube; SIGMA). The PBMCs were washed in PBS, were counted, and were aliquoted to ~1 × 10⁶ cells/cryovial. The cells were pelleted by centrifugation and then were stored at −70°C. Plasma was also collected from the ficoll-hypaque gradient and was stored at −70°C. Saliva was obtained from cotton plugs that had been chewed by individuals for 1–2 min. The plugs were placed in collection tubes (Starstedt) and were spun at low speed; the liquid was then transferred to cryovials and was stored at −70°C. First-void urine samples were obtained in sterile collection cups. Urine samples (10–12 ml each) were spun at low speed (2000 g) for 10 min at room temperature, to pellet cellular material, and the supernatant was separated from the pellet and was stored at −70°C. The pellet was washed once with PBS, was repelleted, and was stored at −70°C.

Extraction/purification of DNA. PBMCs, saliva, and urine samples were processed by use of a standard proteinase K-phenol extraction protocol [19]. In brief, saliva samples ≤500 μl were incubated for 2 h at 55°C with proteinase K (Fisher), were extracted by use of phenolchloroform, and the aqueous layer was precipitated by isopropanol. PBMCs and urine pellets were digested with proteinase K, in a total volume of 1 ml, for 4 h at 55°C. The digests were extracted with 500 μl of Trisbuffered phenol (pH 8.0), and the DNA was precipitated with sodium acetate and was washed once with 70% ethanol. The DNA pellets were then resuspended in Tris-EDTA buffer (pH 8.0) and were stored at −20°C. (Some specimens were initially prepared by use of the QIAamp DNA Blood Mini Kit [Qiagen], but this approach was discontinued when we found that DNA yields were smaller and that the sensitivity of detection of polyomaviruses was decreased.)

Oligonucleotides and polymerase chain reaction (PCR) analysis. EBV was detected by real-time quantitative PCR with a fluorogenic probe, as described elsewhere [20]. The primers and the probe, for a conserved region of the EBER-1 gene of EBV, were developed by use of primer express software (PE Applied Biosystems). The forward and reverse primers (EBER-UP and EBER-UP) were 5′-TACGTTGCTTGCAGGAGATG-3′ and 5′-CGTCTACGTCTCTCTCAAGC-3′. The fluorogenic probe was VIC-TGCAAAACCTCAGGACCT-ACGCTGC-TAMRA. The PCR reaction was performed by use
of the Taqman PCR kit (PE Applied Biosystems). In brief, 500 ng of DNA extracted from either PBMCs or saliva was added to a PCR mixture containing the following: 10 mM Tris (pH 8.3); 50 mM KCl; 10 mM EDTA; 5 mM MgCl₂; 100 μM dATP, dCTP, dGTP, and dTTP; 0.2 μM each primer; 0.1 μM fluorescent probe; and 1.25 U of AmpliTaq Gold. Samples with a known template amount were run during the reaction, to generate a standard curve of Ct versus LogN (copyN/known template amount were ran during the reaction, to generate a standard curve of Ct versus LogN) (N = original copy number in standard). The standards were prepared by use of IB4 cells, which contain 2 EBV genome copies/cell. EBV-negative DG75 cells were used to equilibrate all standards to a total mass of 500 ng. A dilution series was made with decreasing amounts of IB4 DNA (100, 50, 10, 5, 1, 0.5, and 0 ng); these amounts correspond to 20,000, 10,000, 2000, 1000, 200, 100, and 0 copies of EBV, respectively. The limit of detection of the assay was ∼10 EBV copies/500 ng of total PBMC DNA. Because of the variable nature of DNA obtained from saliva, and as a confirmation of sample quality, all samples were also tested for amplification of the β-actin gene. Primers and conditions for real-time PCR of β-actin were followed, according to the manufacturer’s recommendations (PE Applied Biosystems). CMV was detected by use of the Digene Sharp Signal system (Digene Diagnostics) [10], according to the manufacturer’s recommendations. The limit of detection of this CMV assay was 10 genome copies/reaction. PCR for CMV DNA was performed on urine samples from 4 of the 7 collection times.

Oligonucleotide primers used for both PCR and DNA sequence analysis of polyomaviruses have been described elsewhere [14, 21–24]. DNA samples were tested for suitability for amplification, by use of primers specific for a fragment of the human β-hemoglobin gene (primers AG1 and AG2) [25]. If specific PCR products were formed, those samples were considered to contain adequate amounts of cellular DNA. However, the detection of viral DNA in urine was not dependent on the level of amplifiable cellular DNA, so the analysis of the β-hemoglobin gene in urine samples was discontinued partway through the study. Plasmids pBK-Dunlop (obtained from the American Type Culture Collection) and pJC-MAD-1 (a gift from M. Sullivan) served as positive-control templates. PCR amplifications were performed either in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer) or in a PTC-200 Peltier Thermal Cycler (MJ Research), for a total of 45 denaturation, annealing, and extension steps, by use of temperatures specific for each primer pair. Each PCR reaction contained either 0.5–1 μg of PBMC DNA (∼8 × 10⁻⁴–1.6 × 10⁻⁴ cell equivalents) or DNA extracted from a urine pellet (equivalent to 2–2.4 mL of urine), in a 100-μL reaction volume. PCR products were analyzed on a 2% ethidium-bromide–stained agarose gel. The limit of detection of the PCR assay for polyomaviruses was ∼10 genome copies/500 ng test DNA.

**Polyomavirus DNA sequence analysis.** DNA sequencing reactions were performed by use of a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB). Polyomavirus PCR products were visualized by electrophoresis on a 2% agarose gel. If the reactions were weak, products were cloned by use of the TOPO TA cloning kit (Invitrogen) and then were sequenced. Polyomavirus PCR products that gave strong reactions were sequenced directly after treatment with shrimp–alkaline phosphatase and exonuclease I.

**Culture from urine.** Urine supernatants were used for viral culture. In brief, the supernatant fluid was passed through a 0.45-μm filter, and the filtrate was inoculated into subconfluent COS-7 cells grown in T-75 (75-cm²) flasks [26]—cells that have been reported to support the growth of many JCV strains and some BKV strains. Dulbecco’s MEM (DMEM) containing 10% fetal calf serum was used to grow the cells until confluency was reached, and, thereafter, DMEM containing 3% fetal calf serum was used to maintain the cells. Cells were split once, at a ratio of 1:5, at 5 days postinoculation, and were fed with fresh media every 3 days. Cells were observed for 2 months, for the presence of cytopathic effects, and were tested by PCR, for the presence of JCV and BKV, as described above.

**Detection of EBV-specific and CMV-specific antibodies.** IgG antibodies specific for EBV viral-capsid antigen and EBV nuclear antigen were detected by indirect immunofluorescence assays [20, 27], as were antibodies against CMV [10].

**Statistical analysis.** Pearson correlation analysis was used to test the relationship between the frequency of EBV shedding in saliva and that in blood. EBV levels were transformed to log₁₀ scale before analysis. Fisher’s exact test was used for comparisons of the percentages of subjects positive for JCV and CMV. Cochran’s Q test was used to compare the seasonal frequencies of shedding of EBV and JCV. Analysis of variance for
repeated measures was used to compare mean seasonal levels of EBV. SPSS software was used for all statistical analyses.

**RESULTS**

The demographic data for the 30 adult volunteers enrolled in the study are presented in Table 1. No major acute illnesses were diagnosed during the study period, but there was a high frequency of respiratory and/or flulike symptoms reported by different volunteers during the periods of March–May 1999 and October–December 1999 ( ).

**EBV.** All the subjects in this study possessed EBV-specific IgG antibodies, a finding that suggests prior infection with EBV (data not shown). The mean EBV DNA load in saliva and in PBMC samples, in each collection period, is presented, according to frequency of detection, in Table 2. Every person in the cohort shed EBV in saliva at least once during the 14-month study period; control values were set equal to 1.0, so that log$_{10}$ values were defined as low viral shedders if they had shed a given virus on 0–1 occasions, as moderate shedders if they did so on 2–3 occasions, and as high shedders if they did so on 4–6 occasions, over the 14-month study period (1 individual in this group provided only 4 blood samples, and another individual provided only 5 blood samples).

**Table 2.** Mean loads of herpesvirus Epstein-Barr virus (EBV) DNA in saliva and in peripheral blood mononuclear cells (PBMCs) in healthy volunteers, by quantitative polymerase-chain-reaction analysis.

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<tbody>
<tr>
<td><strong>Saliva</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (5)</td>
<td>0</td>
<td>0</td>
<td>0.43 (0.95)</td>
<td>0</td>
<td>2.42 (1.70)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Moderate (10)</td>
<td>0.89 (1.92)</td>
<td>0.92 (1.46)</td>
<td>1.45 (1.79)</td>
<td>1.19 (1.59)</td>
<td>1.01 (1.21)</td>
<td>1.35 (1.67)</td>
<td></td>
</tr>
<tr>
<td>High (15)</td>
<td>1.59 (1.96)</td>
<td>1.92 (1.76)</td>
<td>3.29 (2.02)</td>
<td>2.59 (1.75)</td>
<td>2.16 (1.76)</td>
<td>3.01 (1.66)</td>
<td>1.63 (1.96)</td>
</tr>
<tr>
<td><strong>PBMCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (5)</td>
<td>0</td>
<td>0</td>
<td>0.38 (0.87)</td>
<td>0</td>
<td>0.10 (0.21)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Moderate (10)</td>
<td>0.06 (0.21)</td>
<td>0.40 (0.72)</td>
<td>0.21 (0.42)</td>
<td>0.18 (0.58)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>High (15)</td>
<td>0.52 (0.89)</td>
<td>0.82 (0.92)</td>
<td>0.31 (0.90)</td>
<td>0.42 (0.90)</td>
<td>0.15 (0.45)</td>
<td>0.20 (0.53)</td>
<td></td>
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</table>

**NOTE.** Data are mean (SD) log$_{10}$ EBV genomes/500 ng PBMCs or saliva DNA. Values below the limit of detection (<10 EBV copies/500 ng of PBMC DNA or saliva DNA) were set equal to 1.0, so that log$_{10}$ = 0 for those values. Individuals were defined as low viral shedders if they had shed a given virus on 0–1 occasions, as moderate shedders if they did so on 2–3 occasions, and as high shedders if they did so on 4–6 occasions, over the 14-month study period (1 individual in this group provided only 4 blood samples, and another individual provided only 5 blood samples).

**CMV.** Nineteen (63%) of the subjects in this study tested positive for CMV antibody; however, 1 of the antibody-negative subjects shed CMV on 1 occasion, a finding that suggests that the CMV antibody assay may not identify all infected individuals. Only 4 (13%) of the volunteers shed CMV in urine; the shedding appeared to be age dependent, since all the individuals who shed CMV were <40 years old ( ) (table 3).

**Polyomaviruses.** Extracts of urine pellets from volunteers were tested for the presence of JCV and BKV. JCV viruria was detected at least once in urine samples from each of 14 (47%) of the subjects (table 3). All urine samples were negative for BKV. JCV shedding was age dependent, occurring significantly more frequently in subjects ≥40 years old ( ) (table 3).

**Table 3.** Sex and age of JC virus (JCV)–positive and cytomegalovirus (CMV)–positive individuals, among healthy volunteers.

<table>
<thead>
<tr>
<th>Group</th>
<th>JCV Positive$^a$</th>
<th>P</th>
<th>CMV Positive$^b$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Positive$^a$</td>
<td></td>
<td>Positive$^b$</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6/15 (40.0)</td>
<td>.72</td>
<td>2/15 (13.3)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Male</td>
<td>8/15 (53.3)</td>
<td></td>
<td>2/15 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14/30 (46.7)</td>
<td></td>
<td>4/20 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>&lt;40</td>
<td>3/13 (23.1)</td>
<td>&lt;.03</td>
<td>4/13 (30.8)</td>
</tr>
<tr>
<td>Age, years</td>
<td>≥40</td>
<td>11/17 (64.7)</td>
<td>0/17</td>
<td>4/20 (13.3)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%), unless otherwise noted. When the JCV results are limited to the same 4 urine samples that were tested for CMV, 1 fewer females in the <40 age group are JCV positive (2/13 [15.4%] for the <40 age group and 13/30 [4.33%] for the total calculation). In the limited analysis, there were still no effects related to sex (P = .46), and JCV shedding was significantly age related (P < .01).

$^a$ In ≥1/7 urine samples.

$^b$ In ≥1/4 urine samples.
Figure 1. A, Archetypal regulatory-region sequence amplified by primers JC1 and JC2, in JC virus (JCV) isolate CY (GenBank AB038249). JC1- and JC2-primer binding sites are underlined (straight line) and identified. Tst-1, cre, Sp1, NF-1, TAR, p53, and Ap1 binding sites also are underlined (straight line or wavy line) and are identified above the JCV sequence. The portion of the JCV origin of replication (Ori) present within this sequence is indicated (horizontal arrows pointing left). Subdivisions (25 bp, 23 bp, 55 bp, 66 bp, and 18 bp) of the regulatory region, according to Ault and Stoner [31], are identified below the JCV sequence.

B, Regulatory region of JCV reference strain MAD-1. The regulatory region is depicted in the customary fashion, showing tandem 98-bp repeats. The JCV–MAD-1 regulatory region is clearly different from that of JCV-CY.

C, Nucleotide sequences of archetypal JCV regulatory regions detected in urine from healthy volunteers. A schematic representation of the JCV isolate’s CY regulatory region is shown, depicting the sequence described in panel A. Nucleotide deletions detected in some of the specimens are shown; each sequence described is from a different individual.

Urine (as defined in table 2) represented 14%, 36%, and 50% of the 14 positive individuals, respectively. Six (86%) of 7 individuals who shed high levels were ≥40 years old (P = .003). Urine samples from 2 collection times (November 1998 and May 1999) were tested for the presence of infectious virus, by culture of COS-7 cells. In all cases in which viral DNA was detected directly in urine samples by PCR, JCV DNA was detected in cultured-cell extracts by PCR (data not shown). JCV was not detected in cell lysates exposed to urine samples that had tested negative by PCR. In contrast, all the PBMC samples tested negative for JCV and BKV (limit of detection, ∼10 viral genome equivalents/500 ng of test DNA). We experimented by
using larger quantities of PBMC DNA (≤10 μg of test DNA/PCR analysis), and again we failed to detect any polyomavirus DNA, although all samples were suitable for PCR analysis, as evidenced by the amplification of the β-hemoglobin gene (data not shown).

**Molecular typing of JCV.** Many genotypes of JCV, as well as of viruses with different regulatory regions, are known to exist. Molecular typing is useful in natural-history studies. Polyomavirus regulatory regions contain enhancer and promoter elements and the viral origin of DNA replication; all the known polyomaviruses can be distinguished on the basis of their regulatory-region sequences. DNA sequence analysis was performed on PCR products obtained, by use of the JC1 and JC2 primers, from selected samples; the results verified viral identity (as JCV) and showed the genetic architecture of the regulatory region of the VP1 gene. Repeated shedding of the same viral strain was shown for volunteers 2, 9, 14, and 16, with 2 or 3 different collection times, and for the positive control shedder, with 7 different specimens.

<table>
<thead>
<tr>
<th>JCV genotype</th>
<th>Designation(s) of volunteer(s)</th>
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<tbody>
<tr>
<td>1A</td>
<td>14</td>
</tr>
<tr>
<td>1B</td>
<td>23</td>
</tr>
<tr>
<td>2A</td>
<td>9, 15, 16, 18, and positive-control shedder</td>
</tr>
<tr>
<td>2B</td>
<td>1, 2</td>
</tr>
<tr>
<td>2C</td>
<td>25</td>
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<td>4</td>
<td>24</td>
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</table>

**NOTE.** To type and subtype JCV strains, we used the sequence of a 215-bp region of the VP1 gene. Repeated shedding of the same viral strain was shown for volunteers 2, 9, 14, and 16, with 2 or 3 different collection times, and for the positive control shedder, with 7 different specimens.

**DISCUSSION**

This is the first longitudinal study that we are aware of that evaluates, over time, both viral reactivation and shedding of several different persistent viruses, in immunocompetent individuals. The results show that the dynamics of virus shedding and reactivation vary among individuals and that there is not a coincidence of reactivation of viruses within a given individual at any particular time. These findings suggest that the reactivations of latent/persistent viruses among immunocompetent adults are functions of (1) the specific biological features of a virus, (2) the possible presence of local stimuli for activation of virus in infected tissues, and/or (3) specific facets of the immune system that are important in the control of each particular virus. Indeed, one interesting feature of viral activity observed in the present study is an increase in frequency and mean viral loads of EBV shedding in saliva during spring and fall months. The reasons for this increase are unclear—it could be due to a number of factors. One possible explanation is that these months represent the peak allergy seasons in Houston; the increased presence of allergens might contribute to either an increase in oral secretions or an influx of immune cells, including B cells, that could result in increased shedding of seasonal infections with JCV, and not reinfection.

**Seasonal variation in virus shedding.** We observed seasonal effects on shedding, for both EBV (figure 2A) and JCV (figure 2C). EBV shedding in saliva fluctuated during the course of the year, with higher shedding during spring (March–May) and fall (October) (P = .02) (figure 2A). There was a similar seasonal difference in the detection of EBV DNA in blood, with higher frequencies during the winter (November–January) and spring (P = .01) (figure 2B). JCV shedding in urine was more frequent during the fall and winter (P = .05) (figure 2C). These observations suggest that EBV shedding and JCV shedding are not “in phase.”

**Lack of correlation, of shedding, between virus groups.** The frequencies with which the viruses were shed simultaneously during this longitudinal study are shown in table 5. Among all samples tested, EBV and JCV shedding approached 51% and 27%, respectively, whereas CMV shedding was ~3%. EBV and JCV were shed simultaneously only 14% of the time. EBV and CMV were shed simultaneously in only 2 instances, and JCV and CMV were never shed simultaneously. All 3 viruses were never shed simultaneously by a healthy volunteer. Using the Kappa statistic for concordance, we found no agreement, other than what would be expected by chance, for any virus combination (P ≥ .50). During the course of this study, individuals who were high shedders of one virus (either EBV or JCV) were not necessarily frequently positive for the other. There was no correlation between EBV shedding and JCV shedding (r = 0.04; P > .80).

**Table 4. Shedding of JC virus (JCV) genotypes in the urine of healthy adult volunteers.**

<table>
<thead>
<tr>
<th>JCV genotype</th>
<th>Designation(s) of volunteer(s)</th>
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<tr>
<td>1A</td>
<td>14</td>
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<tr>
<td>1B</td>
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<tr>
<td>2A</td>
<td>9, 15, 16, 18, and positive-control shedder</td>
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<tr>
<td>2B</td>
<td>1, 2</td>
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<td>25</td>
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<tr>
<td>4</td>
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**NOTE.** To type and subtype JCV strains, we used the sequence of a 215-bp region of the VP1 gene. Repeated shedding of the same viral strain was shown for volunteers 2, 9, 14, and 16, with 2 or 3 different collection times, and for the positive control shedder, with 7 different specimens.
either EBV-infected epithelial cells or infected trafficking B cells. Alternatively, these months might represent periods in which infections by respiratory pathogens abound, and this increased presence of pathogens might contribute to an increase in viral replication or virus release from the oral cavity [32]; in fact, volunteers reported increased respiratory symptoms during spring and fall months. We do not understand the difference between the frequency of EBV shedding in saliva and the detection of EBV DNA in PBMCs, but we speculate that it might reflect the difference in target cells involved (epithelial vs. lymphoid). Since external cofactors influence virus reactivation, different patterns of virus shedding are to be expected under different circumstances. We do not believe that the discrepancies in our findings are due to sampling errors, because samples were randomly collected from each of the subjects over a 3-day period during each of the collection times, and negative controls analyzed in parallel were routinely negative, which rules out laboratory contamination of the specimens. Furthermore, the specimens from a given collection cycle were not batch processed, and the same investigators prepared and analyzed the samples throughout the entire study; therefore, we do not believe that technical variations are responsible for the observed differences.

In general, the present study’s reported frequency of detection of EBV in saliva is similar to other studies’ detection rates, which ranged from 22% to 90% [33–37]; over a 12-month period, our results ranged from 32% to 73%. We are unaware of any other studies that have used PCR to quantitatively measure, over time, EBV levels in saliva in healthy adults. Therefore, in this regard, our data are novel (table 2). One study, using real-time PCR, reported that EBV loads in saliva in 12 healthy individuals ranged from 2 to 6523 copies/µg DNA, a finding that is similar to our results [38]; in that study, the frequency of detection of EBV in blood ranged from 0 to 30%, which is slightly lower than the range (0–54%) that had been reported in earlier studies [36, 39, 40]. The PCR primers used in our study recognized well-conserved sequences in the EBER-1 gene, whereas other studies used several different probes, including probes that amplify regions in the major internal repeats (e.g., IR1 in BamW) and in the EBNA2, BNRF1, and BALF5 genes [36, 41, 42]. It is possible that primers to those genes amplify their respective sequences more readily than do our EBER-1 primers. It is difficult to compare the levels of EBV detected in PBMCs in our study to the levels detected in other studies, because of differences in technical sensitivity, in the standard used, and in expression of results. Nonetheless, other studies using real-time PCR have reported mean EBV copy numbers that ranged from 0 to 10^{12} copies/µg DNA [38, 41], copy numbers that are similar to our results. However, in a given subject, we were unable to detect any correlation between either viral shedding frequency or virus load in saliva and the presence of EBV in PBMCs, a finding similar to one made elsewhere [36]. Perhaps, the factors governing EBV reactivation in the oropharynx are different from those governing virus loads in the blood.

Our results confirm the shedding of JCV by healthy humans as they approach or surpass 40 years of age [23, 43]. In the present study, an important finding is that PCR-detected JCV DNA in urine represents an infectious form of the virus, since JCV DNA was detected in COS-7 cells inoculated with urine samples from healthy volunteers. Urinary JCV contained archetypal regulatory regions, a finding that is consistent with the notion that the transmitted form of the virus has this arrangement [28, 44, 45]. Furthermore, within a given individual, the JCV sequences that were analyzed in multiple samplings over the course of this study remained stable. These findings support the notion that JCV infections are persistent [46]. However, only partial correlation was observed when JCV types were compared with an individual’s race or ethnic group [29, 47, 48]. For example, whereas JCV subtype 2A is ordinarily carried by Asians, the present study found that 4 white individuals shed type 2A strains. A likely reason for this is that the American population is diverse and that there would therefore be ample opportunity for children or young adults to acquire primary JCV infections from persons of different ethnic backgrounds. Although the present study suggests that JCV shedding in urine is more frequent during spring and fall months, the underlying mechanism responsible for this seasonal variation in virus reactivation is not known. The apparent seasonal fluctuation in shedding of JCV, as well as that in EBV (discussed above), may indeed influence the results of single-collection studies involving these viruses.

### Table 5. Lack of correspondence between shedding of Epstein-Barr virus (EBV), shedding of JC virus (JCV), and shedding of cytomegalovirus (CMV), in healthy volunteers.

<table>
<thead>
<tr>
<th>Virus(es)</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>100/198  (50.5)</td>
</tr>
<tr>
<td>JCV</td>
<td>55/203  (27.1)</td>
</tr>
<tr>
<td>CMV</td>
<td>4/116   (3.4)</td>
</tr>
<tr>
<td>EBV and JCV</td>
<td>28/198  (14.0)</td>
</tr>
<tr>
<td>EBV and CMV</td>
<td>2/116   (1.7)</td>
</tr>
<tr>
<td>JCV and CMV</td>
<td>0/116</td>
</tr>
<tr>
<td>EBV, JCV, and CMV</td>
<td>0/116</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of samples (%). No. of volunteers is 30. Specimens were collected on 7 occasions from November 1998 to December 1999 (1 volunteer provided 4 collections, 4 volunteers provided 6 collections, and 25 volunteers provided 7 collections). Urine samples were tested for JCV (n = 4–7 samples/volunteer) and CMV (n = 4 samples/volunteer); saliva samples were tested for EBV (n = 4–7 samples/volunteer).
Figure 2. Seasonal variation of shedding in Epstein-Barr virus (EBV) and of JC virus (JCV) and in EBV detection in blood, in healthy adults. A, EBV shedding in saliva. The percentage of healthy volunteers (n = 30) with saliva samples positive, by PCR, for EBV DNA, at the indicated collection times, over a 14-month period, are shown. B, EBV detection in PBMCs. Blood samples were collected from the same volunteers as are described in panel A; the percentages of samples positive for EBV DNA over time are shown. C, JCV shedding in urine. Urine samples were collected from the same volunteers as are described in panel A; the percentages of samples positive for JCV DNA over time are shown.

In this study, no urine samples tested positive for BKV. Perhaps, in urine of healthy adults, the prevalence and quantity of BKV are lower than those of JCV. These findings are consistent with the results of other studies, which also failed to detect BKV in urine of healthy adults [13, 22, 49–51]. However, differences in the methodology used to analyze polyomaviruses in urine might account for the lack of detection of BKV. We have determined that the sensitivity of polyomavirus PCR assays is lower when the urine specimens are extracted by use of a commercial kit (data not shown). Two reports, using the same type of kit, have documented similar results for CMV [52] and for adenovirus [53]. However, BKV was not detected in specimens that we prepared by conventional methods, whereas the relative amount of PCR-amplified JCV-specific products increased. Thus, the sensitivity of our PCR methods was improved, yet only JCV was detected in urine samples.

No polyomaviruses were detected in blood samples. This suggests either that polyomaviruses are seldom present in PBMCs of immunocompetent subjects or that the virus load is below the levels detectable by our assays. Our inability to detect JCV in PBMCs of adult volunteers is consistent with the findings of other studies [54–56]. It remains unclear why there is variability in the detection of JCV in PBMCs of healthy individuals.

In conclusion, our study has shown that the levels of shedding and reactivation of latent/persistent viruses vary among individuals and by season. More important, there was not a coincident reactivation of multiple viruses within a given subject. These findings support the hypothesis that immunocompetent individuals control latent/persistent viruses independently.

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References


