Prevalence and Cellular Reservoir of Latent Human Herpesvirus 6 in Tonsillar Lymphoid Tissue

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Abstract

There are few studies that examine prevalence, quantity, and cellular proclivity of latent human herpesvirus 6 (HHV-6) in healthy populations. We examined 69 tonsils with paired blood specimens from children without evidence of acute infection. By polymerase chain reaction (PCR), HHV-6 was detected at low levels in 100% of tonsils and 39% of blood samples (n = 27), suggesting that prevalence of latent HHV-6 infection is high in children and may be underestimated by PCR analysis of blood. Although HHV-6A and HHV-6B were detected, HHV-6B predominated, being found in 97% of samples (n = 67). Tonsil sections from 7 cases were examined by in situ hybridization using 2 HHV-6 probes and immunohistochemical analysis. Using both in situ hybridization and immunohistochemical analysis, all tissues revealed marked HHV-6-specific staining in the squamous epithelium of the tonsillar crypts and rare positive lymphocytes. We conclude that HHV-6 is present universally in tonsils of children, and tonsillar epithelium may be an important viral reservoir in latent infection.

Since the discovery of human herpesvirus 6 (HHV-6) by Salahuddin and colleagues1 in 1986, the virus has been associated with several disease states, including HIV infection,2 multiple sclerosis,3 postrenal transplant states,4 post–bone marrow transplant states,5 and certain neoplastic disorders including non-Hodgkin lymphomas6,7 and Hodgkin disease.8,9 HHV-6 has been linked causally to the childhood disease exanthem subitum.10 However, HHV-6 infection alone is insufficient for the development of any of these disease states, since 90% of children have been found to have serologic evidence of exposure by age 2.11 As with other members of the herpesvirus family, HHV-6 enters a latent state following acute infection, without subsequent clinical manifestations of disease in immunocompetent people.

HHV-6 is classified into 2 variants, HHV-6A and HHV-6B, with different epidemiologic and clinical manifestations attributed to each.12 The prevalence of the 2 subtypes seems to be dictated predominantly by differences in geographic distribution.

Because of the ubiquitous nature of the virus, there have been recent attempts to find markers to distinguish latent infection from lytic or acute disease. The expression of the U94 messenger RNA transcript has been proposed as a marker for latency.13 Several immediate early transcripts have been proposed as markers for lytic infection. In addition, the levels of virus in circulation may distinguish these 2 viral states, with low levels of virus detected in patients lacking evidence of acute infection.14,15

The goal of the present study was to determine the prevalence of HHV-6 and viral burden levels using the sensitive polymerase chain reaction (PCR) technique in the blood and lymphoid tonsillar tissues in a population of children
with latent infection. Children older than 2 years undergoing elective tonsillectomy, without evidence of an acute viral syndrome, were selected as a source of material for analysis. HHV-6A and HHV-6B variants were delineated to compare prevalence of the 2 variants in this setting. In addition, in situ hybridization (ISH) using probes derived from an immediate early gene (U89) and a putative latent gene (U94) was performed to determine the cell type harboring HHV-6 in the tonsil. Immunohistochemical analysis also was performed to confirm the ISH results.

Materials and Methods

Samples

After institutional review board approval was obtained, fresh tonsillar tissue and EDTA-anticoagulated whole blood were obtained from 69 children undergoing elective tonsillectomy for moderate tonsillary hyperplasia or recurrent streptococcal infection. A portion of each tissue specimen was processed routinely in formalin for light microscopy. DNA was extracted from fresh tonsillar tissue and whole blood drawn immediately before tonsillectomy using the QIAamp Tissue Kit according to the manufacturer’s protocols (Qiagen, Valencia, CA). A negative control consisting only of wash buffer was extracted concomitant with the samples. To confirm successful DNA extraction, samples were amplified by PCR using primers specific for medium chain acyl CoA dehydrogenase, with subsequent ethidium bromide band detection on 8% polyacrylamide gels. Samples found to be deficient of DNA by this analysis were reextracted until successful amplification was achieved.

Amplification Conditions

DNA samples were analyzed for HHV-6 levels by quantitative PCR with primers previously described (HHV-6 Primer Set A). All PCR reactions were performed with 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA) in 1× PCR buffer with a 1.5-mmol/L concentration of magnesium chloride, a 160-µmol/L concentration each of deoxythymidine triphosphate (dTTP), deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP), with a 152-µmol/L concentration of deoxythymidine triphosphate (dTTP), and an 8-µmol/L concentration of digoxigenin-labeled deoxuryridine triphosphate (dUTP; 19 dTTP:1 digoxigenin-labeled dUTP ratio). Twenty copies of a previously described internal calibration standard (ICS), HHVQ-1, were added to each tube along with 10 µL of extracted DNA sample in a 50-µL final volume. The internal control is a synthetic plasmid that contains recognition sites for the HHV-6 primers. PCR amplification of this internal control generates product slightly larger than product derived from the viral template. Amplification reactions initially were heated to 95°C for 2.0 minutes, then processed through 34 cycles of 95°C for 0.5 minute, 60°C for 0.5 minute, and 72°C for 1.0 minute, followed by 72°C for 9.0 minutes in a thermocycler (Gene Amp 9700, Perkin-Elmer, Norwalk, CT). This procedure gives a digoxigenin-labeled 525-base-pair (bp) HHV-6 product and a digoxigenin-labeled 686-bp ICS product in a PCR reaction for which the kinetics and accuracy were validated previously.

Qualitative Enzyme-Linked Immunosorbet Assay Detection of PCR Products

After amplification, DNA was detected using a commercially available PCR enzyme-linked immunosorbet assay kit (ELISA; Dig Detection, Roche Molecular Biochemicals, Indianapolis, IN), which was used according to the manufacturer’s protocol. Reactions were performed in duplicate, using either the biotinylated HHV-6 or the biotinylated ICS probes (described later in the “Materials and Methods” section). In brief, 10 µL of product was denatured with 20 µL of denaturation solution for 10 minutes at room temperature in microcentrifuge tubes. Then, 220 µL of hybridization solution with either a 7-nmol/L concentration of biotinylated HHV-6 or ICS probe was added to a single tube in the duplicate samples. No cross-reactivity was detected with the other herpesviruses using these probes (data not shown). Aliquots of 200 µL from each tube were added to streptavidin-coated wells and incubated for 3 hours at 37°C. The plate was washed 5 times, 200 µL of peroxidase-conjugated antidigoxigenin was added to each well, and the plate was incubated at 37°C for 30 minutes. After extensive washing, 200 µL of substrate was added to each well, and the plate was incubated in the dark at 37°C for 30 minutes. Optical density (OD) was measured on a spectrophotometer at 405 nm. Negative, low (20 copies), and high (10,000 copies) HHV-6 controls were included in each run. The OD value for the negative control (background) was subtracted from each raw OD value. Samples incubated with the HHV-6 probe were called positive for HHV-6 if the OD was more than 0.100 unit above background. Duplicate reactions for each sample also were examined for ICS product detection. If the ICS probe–labeled samples were not more than 0.100 OD above background, the reaction was repeated since this finding suggested failed amplification.

Quantitative PCR ELISA

All samples found positive for the presence of HHV-6 by qualitative ELISA were quantified as follows. Positive samples were reamplified under the same conditions as specified in the preceding text. The amplification products were serially diluted 1:5 in 5 wells for HHV-6 detection and 3
wells for ICS detection and then subjected to ELISA detection as described. The HHV-6 copy number was determined as previously reported.15

**HHV-6 Typing**

The HHV-6 variant A or B was determined in tonsillar tissue by using PCR primers for a region that gives products with different molecular weights for HHV-6A (325 bp) and HHV-6B (553 bp) following amplification.16 PCR reactions were performed with 2.5 U of AmpliTaq polymerase (Perkin-Elmer) in 1× PCR buffer with a 1.5-mmol/L concentration of magnesium chloride; 50 pmol of each primer; a 100-µmol/L concentration each of dATP, dCTP, dGTP, and dUTP; and 10 µL of the DNA in a 50-µL final volume. Amplification reactions were subjected to 10 cycles of 94°C for 3.5 minutes, 60°C for 0.5 minute, and 72°C for 0.3 minute, followed by 20 cycles of 94°C for 0.5 minute, 60°C for 0.5 minute, and 72°C for 0.3 minute, and concluding with 72°C for 10.0 minutes in a thermocycler (Gene Amp 9700). The products were electrophoresed on 5% polyacrylamide gels, stained with ethidium bromide, and visualized by UV light illumination. Some samples did not amplify with these primers and were subjected to a seminested PCR using the same 5’ primer and a different 3’ primer (5’GGTGCTGAGTGATCAGTTTC) just inside the original 3’ primer yielding a 301-bp product for HHV-6A and 529-bp product for HHV-6B. The same amplification conditions were used for the second round of PCR.

**ISH Probe Development**

Two digoxigenin-labeled probes were prepared for ISH from the U89 and U94 genes by PCR. Blast 2.0 searches against GenBank confirmed that both probe sequences are present in HHV-6A and HHV-6B. Primer sequences were 5’-CTTCTGTCCCATCACTGTCAT and 3’-TCAGAGAGTTTGAGATGG for the U89 probe and 5’-GAATGTACCTTCTGTCCCATCACTGTCAT and 3’-TCAGAGAGTTTGAGATGG for the U94 probe. Probes were synthesized separately in PCR reactions with 2.5 U of AmpliTaq DNA polymerase, 1× PCR buffer with a 1.5-mmol/L concentration of magnesium chloride; 50 pmol of each primer; a 100-µmol/L concentration each of dATP, dCTP, dGTP, and dUTP; and 10-µL final volume. Amplification reactions were subjected to 10 cycles of 94°C for 3.5 minutes, 60°C for 0.5 minute, and 72°C for 0.3 minute, followed by 20 cycles of 94°C for 0.5 minute, 60°C for 0.5 minute, and 72°C for 0.3 minute, and concluding with 72°C for 10.0 minutes in a thermocycler (Gene Amp 9700). The products were electrophoresed on 5% polyacrylamide gels, stained with ethidium bromide, and visualized by UV light illumination. Some samples did not amplify with these primers and were subjected to a seminested PCR using the same 5’ primer and a different 3’ primer (5’GGTGCTGAGTGATCAGTTTC) just inside the original 3’ primer yielding a 301-bp product for HHV-6A and 529-bp product for HHV-6B. The same amplification conditions were used for the second round of PCR.

**In Situ Hybridization**

Formalin-fixed, paraffin-embedded, unstained 4-µm sections of tonsillar tissue on coated glass slides were deparaffinized through graded alcohols, rehydrated, and digested with Proteinase K (DAKO, Carpinteria, CA) solution diluted 1:25 in 10× tris(hydroxymethyl)-aminomethane-EDTA disodium dihydrate buffer, pH 8.0, for 10 minutes on a shaker table at room temperature. Subsequently, the sections were hybridized separately with the U89 or U94 probes at a final concentration of 2.5 ng/µL in Brigati Probe Diluent (Research Genetics, Huntsville, AL) at 95°C for 6 minutes followed by 2.5 hours at 37°C. The sections then were washed stringently for 10 minutes in 2× standard saline citrate (SSC) at room temperature and for 10 minutes in 1× SSC at room temperature, followed by a final wash for 10 minutes in 0.1× SSC at 60°C. Sections then were immersed in tris(hydroxymethyl)aminomethane-buffered saline (TBS), pH 7.6, for 5 minutes, then in TBS with 0.1% Triton X (Sigma, St Louis, MO) and 3% bovine serum albumin for 2 hours. Subsequently, sections were incubated at 37°C for 60 minutes with an alkaline phosphatase–conjugated antidigoxigenin antibody solution (Boehringer Mannheim, Indianapolis, IN) diluted according to the manufacturer’s protocol. Sections then were washed sequentially in TBS, pH 7.6, and TBS, pH 9.5, and developed using 5-bromo-4-chloro-indolyl-phosphatase/nitroblue tetrazolium substrate (DAKO) with levamisole. Sections were counterstained with nuclear fast red and examined by light microscopy.

Identification and categorization of infected cell types was based on the morphologic features and size of the cell and on the tissue architecture. This allowed differentiation between lymphocytes and epithelial cells. Both cytoplasmic and nuclear staining were noted. Positive and negative controls were processed concurrently. Negative controls were performed by omitting the probe from the hybridization mixture. Positive control tissue used was from an HHV-6-positive case previously described.9

**Immunohistochemical Analysis**

Four-micrometer tissue sections were deparaffinized and treated with Proteinase K as described for the ISH procedure. Sections were washed between each subsequent solution application with TBS, pH 7.6. Sections were treated with vendor’s concentrations of avidin/biotin Product Purification Kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The reactions yielded probes of 367 bp for U89 and 192 bp for U94. The internal sequences for both probes were checked against GenBank using Blast 2.0 for sequence homologies, and no significant homologies were found among other sequences in the database, including the other 7 human herpesviruses.
(BioGenex, San Ramon, CA) and protein blocker (DAKO) before incubation with a monoclonal primary antibody for HHV-6 (Argene, North Massapequa, NY) at a dilution of 1:50 in 1% bovine serum albumin TBS, pH 7.6, for 60 minutes at 37°C. After washing, the sections were stained with the DAKO LSAB+ System, Alkaline Phosphatase, according to the manufacturer’s protocol. Levamisole was added to the substrate solution before use. The sections were examined after counterstaining with fuchsin (DAKO) and permanent mounting.

Results

The 69 patients ranged in age from 1.9 to 16 years. The group was composed of 38 girls (55%) and 31 boys (45%). Forty-five (65%) were white, 11 (16%) black, 9 (13%) Latin American, and 4 (6%) classified as “other.” All patients were undergoing elective tonsillectomy and were free of fever or other symptoms of viral exanthem such as rash.

HHV-6 was detected by PCR analysis in 69 (100%) of 69 tonsils. Of these, 67 (97%) were fewer than 128 copies per approximately 1 mg of tissue (<80 copies per reaction); the remaining 2 positive specimens contained 128 copies and 243 copies per milligram of tissue. Twenty-seven (39%) of 69 whole blood specimens contained HHV-6 DNA detectable by PCR. All exhibited fewer than 8,000 viral copies per milliliter of whole blood (<80 copies per reaction). Of the 69 positive tonsil samples 2 (3%) were positive for HHV-6A only, 4 (6%) were positive for both HHV-6A and HHV-6B, and 63 (91%) were positive for HHV-6B only.

Since HHV-6 is highly lymphotropic for CD4+ T cells,1 it was anticipated that the lymphocyte subset of cells would be the primary viral reservoir in tonsil. To determine the cellular reservoir, ISH and immunohistochemical analysis were performed on 7 representative tonsils, including those with the highest levels of HHV-6 DNA. See Image 1A for a representative H&E-stained tonsillar section. All tissues had positive cells with both the U89 and U94 probes. In each tissue section there was striking nuclear staining of squamous epithelial cells in tonsillar crypts Image 1B and epithelial cords Image 1C. In contrast, staining was found only rarely in scattered lymphocytes with hybridization diffusely distributed in both nucleus and cytoplasm (insets, Images 1B and 1C). This pattern of hybridization was seen with both probes with approximately the same architectural distribution and density of positive cells. Immunohistochemical staining confirmed extensive viral antigen expression in the epithelium of the tonsillar crypts Image 1D and an occasional lymphocyte.

Discussion

HHV-6 was detected by PCR in whole blood samples of 39% of this healthy pediatric population. In every case, the viral load determined by quantitative PCR was fewer than 8,000 viral genomes per milliliter of blood. Hoang et al14 found HHV-6 DNA in 25% of adult blood donors, with a range in viral titers of 306 to 8,048 viral genome copies per milliliter of blood. Similar results were reported for pediatric emergency department patients with normal blood cell counts, with an overall detection rate of HHV-6 by PCR of 22%.15 We interpret these data as representing the prevalence of HHV-6 detection associated with latent disease for several reasons. None of the patients in the present study had a viral syndrome at the time of surgery and when the blood samples were drawn. In addition, the peripheral blood viral burden was low in each positive case in comparison with the values reported in a study of acute HHV-6 infection. In that study, Chiu et al19 reported more than 400,000 copies of HHV-6 DNA per milliliter of whole blood in 20 pediatric patients with acute seroconversion.

Our study demonstrates universal positivity for HHV-6 DNA in lymphoid tissue from patients between 23 months and 16 years of age. However, a comparison of our findings with other reports of lymphoid tissue detection is difficult since few reports focus on tissue from persons without a pathologic diagnosis (ie, “normals”). Several PCR studies have reported HHV-6 in reactive lymph nodes with positivity rates ranging from 68.4% (n = 19)20 to 9% (of 2 nonhyperplastic and 9 hyperplastic nodes; all samples paraffin extracted).21 The majority of our positive cases (91%) were HHV-6B, which also correlates well with previous literature.22

The data from the present study, taken together with the data published elsewhere, indicate that simple PCR detection of HHV-6 cannot be used as a diagnostic marker of acute infection with pathologic consequences. This is true regardless of whether whole blood samples or lymphoid tissue biopsy specimens are used as source material. However, it seems that quantification of viral burden may be an effective tool for differentiating acute infection from latency. Similar conclusions have been presented for other members of the herpesvirus family, especially quantification of cytomegalovirus in pediatric solid organ transplant recipients.15

The prevalence of HHV-6 in whole blood may not accurately reflect positivity in tissue samples. In the present study, while HHV-6 was detected in only 39% of blood samples, it was detected in 100% of the tonsils derived from the same patients. The prevalence in tonsillar tissue was confirmed in a subset of samples by both ISH and immunohistochemical analysis. Thus, studies relying solely on whole...
blood detection as a means of detecting latent infection may underestimate the true prevalence of HHV-6 in the patient population. In our study, 60% of cases would not have been identified using whole blood PCR analysis alone.

Two different probes were selected for ISH in an effort to identify a method to distinguish between latent and lytic infection. The probes selected were quite sensitive in that they both identified HHV-6 in all 7 cases evaluated. The probes stained the cytoplasm of rare lymphocytes, thereby suggesting that messenger RNA was being detected or that viral DNA was located in the cytoplasm of these cells. Cyttoplasmic localization of HHV-6 DNA by ISH staining has been reported in at least 2 previous articles.23,24 Both of our probes stained all tonsils presumably representing latent infection. In addition, both probes also detected cells in our positive control sample, a case of fatal HHV-6 infection in a 6-month-old, that presumably represents a lytic infection. Thus, the ISH method described herein does not seem to be effective for distinguishing latent from lytic infections. Since PCR-derived DNA probes were used, it is possible that the
probes were detecting both HHV-6 RNA and DNA. Further studies are required to resolve this issue.

Perhaps the most striking finding reported herein is that the majority of HHV-6 detected by ISH and immunohistochemical analysis was present in epithelial cells. Epithelial staining in these tonsils by ISH was nuclear, quite prominent, with no evidence of an inflammatory infiltrate. There have been reports of in vitro infection of epithelial cells, as well as reports of in vivo epithelial cell infection by HHV-6 in renal tubular epithelial cells. To our knowledge HHV-6 infection of the squamous epithelium in tonsillar crypts has not been reported. Our findings also correlate well with previous reports of HHV-6 DNA in the saliva of immunocompetent people.

Immunohistochemical staining also identified both reactive lymphoid and squamous epithelial cell staining in all cases examined. By subjective criteria, the number of cells detected was less than the number detected by ISH, but the basic pattern was similar. This suggests that the ISH approach presented herein is more sensitive for the detection of HHV-6 in infected cells.

We purport that our findings represent latent levels of HHV-6 in tissue and blood in the pediatric population and that tonsillar tissue may represent the viral burden in hematopoietic tissue in general. Our findings support the hypothesis that quantitative viral burden analysis using PCR techniques and ISH will give more meaningful results to define pathologic levels associated with HHV-6 disease. Finding low levels of HHV-6 in tissue and blood, even when associated with specific disease states, is most likely coincidental. Detection of HHV-6 in blood by PCR may underestimate the overall prevalence of this virus in the human population. Lymphoid tissue biopsy may provide a more accurate measure of prevalence. Finally, our data suggest that tonsillar squamous epithelial cells harboring HHV-6 may provide an important repository for the virus during latent infection.

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References


