Association of human herpesvirus 6 subtypes with symptomatic apical periodontitis

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Objective. The occurrence of human herpesvirus (HHV) 6 subtypes A and B in apical periodontitis was determined. The relationship of HHV-6 subtypes to other disease associated herpesviruses, i.e., Epstein-Barr virus (EBV) and human cytomegalovirus, was also investigated.

Study design. Forty apical periodontitis samples (17 symptomatic and 23 asymptomatic) and 40 healthy pulp control samples were collected. Nested polymerase chain reaction was used to detect HHV-6 DNA.

Results. HHV-6 DNA was observed in significantly higher frequencies in apical periodontitis samples than in control samples (20% vs. 2.5%; \( P = .03 \)). Further classification of apical lesions revealed that subtype B of HHV-6 was significantly associated with large-sized and symptomatic lesions (\( P < .01 \)). Thirty-one apical lesions (77%) harbored \( \geq 1 \) of the tested herpesviruses: EBV was the most frequent herpesvirus (72.5%) in apical periodontitis, followed by HHV-6 (20%).

Conclusion. Our findings suggest that EBV and HHV-6B infections can be associated with symptomatic apical periodontitis. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:401-406)

Findings within the past 10 years have established a significant association of certain human herpesviruses with apical periodontitis.1-3 A number of earlier studies focused on the presence of different herpesviruses: Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) were frequently detected in apical periodontitis.1,4 On the other hand, herpes simplex virus and varicella zoster virus rarely occur in apical lesions.2,5 The occurrence of human herpesvirus (HHV) 6 in marginal periodontitis is well documented, similarly to that of EBV and HCMV6-8, however, the occurrence of HHV-6 in apical periodontitis has not been reported.

HHV-6 is highly prevalent in European countries and the USA, as evidenced by seropositivity rates from 75% to 95% in both adults and children.9 Primary infection occurs in early childhood as an asymptomatic infection or as a febrile syndrome with maculopapular rash, named roseola infantum (also known as exanthem subitum or sixth disease). Primary infection is followed by the state of viral latency in the infected host. Reactivation, which can occur spontaneously, results in increased production of viral antigens, which are immediately targeted by cell-mediated immunity in immunocompetent hosts. Nevertheless, cell-mediated immunity can be impaired generally or locally by several factors, e.g., fever, drugs, tissue trauma, stress, and infections.

HHV-6 has 2 subtypes, A and B, which exhibit different biologic features and disease associations. The primary target cells are the CD4 T lymphocytes for both subtypes, but HHV-6A can replicate also in CD8 T cells and natural killer cells.10,11 Monocytes, macrophages, dendritic cells, and bone marrow progenitor cells (CD34+) serve as sites of latent infections for both subtypes.12-14 Subtype B is more commonly associated with febrile illnesses during the first 2 years of age.15 In contrast, HHV-6A seems to play a role in neurologic or immunologic diseases.16

In the present study, we investigated the occurrence of HHV-6A and HHV-6B in apical periodontitis and analyzed their relationship with the clinical manifestation of the disease. The moderate occurrence rates detected in this study, together with our previous data on EBV and HCMV,17 provide an overview of pathogenic herpesviruses in apical periodontitis.
Collection and classification of specimens

A total of 40 apical periodontitis samples were collected from 36 patients (age 18-80 years, mean age 49 years) and 40 healthy pulp samples from 25 patients (age 17-29 years, mean age 23 years). Patients were seeking dental care at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Debrecen. The local Ethical Committee approved the study (approval no. 2885-2008). Patients filled informed consents and they were anonymously coded for identification. Patients with apical periodontitis also filled questionnaires, which contained questions about general diseases, medications, history of the involved tooth, and related symptoms.

The inclusion criteria for patients were as follows: individuals in good health condition (American Society of Anaesthesia level I or II) with no severe systemic disease, history of symptoms of the affected tooth, but requirement for surgical apicoectomy because of the failure of conventional root canal therapy. The history of symptoms and the radiographs showing periapical radiolucent area indicated that the patients suffered from chronic apical periodontitis. Patients with either poor general status or systemic diseases or periodontally involved teeth (probing depth >4 mm) were excluded.

Based on the radiographic size of the periapical lesion, samples were divided into 2 subgroups: ≥5 mm (large) and <5 mm (small) diameters of lesions. Samples with apical periodontitis were divided further into symptomatic and asymptomatic groups according to the symptoms of the involved teeth. Symptomatic lesions were characterized by acute pain, discomfort on biting, or sensitivity by percussion or palpation at the apical region of mucosa. The asymptomatic lesions did not have any clinical symptoms with the exception of periapical radiolucent area on radiographs.

The surgical removal of apical periodontitis lesions and impacted molars was done as follows. Before being administered local anesthetics, patients rinsed their mouth with 0.2% chlorhexidine mouthwash for 1 minute, and the teeth, gingiva, and mucosa of the sample area were washed with 0.2% chlorhexidine. Using a sterile no. 15 blade, gingival incisions were extended 1 or 2 teeth mesially from the involved tooth followed by a vertical releasing incision. A full-thickness mucoperiosteal flap was made. In case of apicoectomy, the periapical lesion was exposed with a sterile round bur using sterile saline solution as coolant. A sterile curette was used to obtain the periapical sample, which was put into a sterile Eppendorf tube with RNAlater RNA stabilization reagent (Applied Biosystems, Foster City, CA) and then immediately frozen to −70°C. The impacted molars were exposed also with sterile round bur using sterile saline as coolant. Each removed tooth was put into a sterile Falcon tube with sterile saline and immediately frozen to −70°C. The extracted tooth was packed separately into a thick sterile plastic bag and was fractured with a metal hammer from outside. Thereafter the plastic bag was placed in the laminar flow box, where it was opened and pulp tissues removed with sterile forceps and files.

Nucleic acid extraction, PCR reactions and statistical analysis

Homogenized tissue samples were divided into 2 portions: one for RNA and the other for DNA isolation. DNA was extracted by High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Total RNA was extracted with TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer’s protocol. The extracted RNA was turned to complementary DNA (cDNA) by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random hexamers. The effectiveness of DNA isolation was controlled with polymerase chain reaction (PCR) detection of human β-globin housekeeping gene.18 For the verification of RNA isolation, PCR detection of a constitutively expressed acidic ribosomal phosphoprotein PO (h36B4) mRNA was used.19

Both rounds of nested PCR amplification for HHV-6 were performed in a final volume of 20 μL using 2× RedTaq PCR reaction Ready Mix (Sigma-Aldrich, St. Louis, MO). The final concentration of each PCR primer was 0.5 μm/L. Primers for HHV-6 DNA amplification were designed to amplify sequences from the immediate early (IE) fragment, which is a conservative regulator region in the HHV-6 genome. The sequences of the outer primers were 5’-TTCTCCA-GATGTGCCAGGGAAAAATCC-3’ and 5’-CATATT-GTTATCGCTTTTCACCTC-3’. The inner primers were 5’-AGTGACAGATCTGGGCGCCCTAATAAC-3’ and 5’-AGGATCTGATGACTCATTTTATTCA-3’. The primer sets are designed to amplify 195 bp from subtype A and 423 bp from subtype B of HHV-6.20 DNA amplification in both rounds was started with an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 2 minutes at 62°C and 3-minute extension at 72°C, and then completed with a final elongation at 72°C for 5 minutes.20 Five microliters of the extracted DNA solution was used as template in the first PCR round and 3 μL PCR product of the first round was used as template in the second round. Positive control samples for HHV-6 subtypes A and B were isolated from
a GS strain and a Z29 strain, respectively (HHV-6 Foundation, Gaithersburg, MD). EBV and HCMV DNA prevalence data are from one of our previous studies. PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide (0.5 μg/mL).

Association was tested between the presence of apical periodontitis lesion and human herpesvirus 6 infection using Fisher exact test owing to low (N=5) number of observations in some cells of the 2 × 2 contingency tables. Logistic regression was applied to determine how the severity of disease (asymptomatic-symptomatic) and the size of lesion (small-large) correlate with the presence of the virus. Significance was accepted for both statistical tests at the 5% level.

RESULTS

Forty samples with apical periodontitis and 40 impacted third molars used as healthy control samples were included in this study. HHV-6 DNA was observed in significantly higher frequency in apical periodontitis than in healthy pulp (8/40 vs. 1/40; P = .03). HHV-6 subtype A and subtype B were present in equal numbers of apical periodontitis lesions: 4 of the 40 samples harbored HHV-6A and 4 other samples contained HHV-6B; none of the samples had dual infection with both subtypes (Fig. 1). Six of these HHV-6–positive DNA samples (2 with subtype A, 4 with subtype B) were sequenced, which confirmed the results obtained by the type-specific PCR method. There was only 1 HHV-6–positive control sample, which was infected by subtype A (Table 1). We could not detect HHV-6 mRNA expression in either pathologic or control samples (data not shown).

Lesions with apical periodontitis were classified according to the radiologic size of periapical bone destruction: 19 lesions were <5 mm (small) and 21 ≥5 mm (large). There was an increased occurrence of HHV-6 in both large-sized (21) and small-sized (19) lesions compared with healthy control samples (40). Logistic regression analysis revealed a significant correlation between the lesion size and HHV-6 infection (P = .018), i.e., increasing lesion size was associated with an increasing frequency of HHV-6 occurrence. There was a characteristic subtype distribution: all 4 HHV-6B infections were detected in large-sized lesions, whereas 3 of the 4 HHV-6A infections were present in small-sized lesions (Table I).

Samples with apical periodontitis were divided further into symptomatic (n = 17) and asymptomatic (n = 23) groups according to the symptoms of the involved teeth. Both symptomatic and asymptomatic lesions contained HHV-6 DNA at increased frequency (5/17

Table I. Prevalence of human herpesvirus 6 (HHV-6) subtypes, human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) in apical periodontitis

<table>
<thead>
<tr>
<th></th>
<th>HHV-6A</th>
<th>HHV-6B</th>
<th>HCMV</th>
<th>EBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>4/40</td>
<td>0/40</td>
<td>4/40</td>
<td>1/40</td>
</tr>
<tr>
<td>Apical periodontitis (n = 40)</td>
<td>4/40</td>
<td>4/40</td>
<td>4/40</td>
<td>29*</td>
</tr>
<tr>
<td>Small-sized (&lt;5 mm)</td>
<td>4/19</td>
<td>4/19</td>
<td>4/19</td>
<td>9*</td>
</tr>
<tr>
<td>Asymptomatic (n = 15)</td>
<td>2/15</td>
<td>0/15</td>
<td>1/15</td>
<td>9*</td>
</tr>
<tr>
<td>Symptomatic (n = 4)</td>
<td>1/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Large-sized (≥5 mm)</td>
<td>4/21</td>
<td>0/21</td>
<td>2/21</td>
<td>6*</td>
</tr>
<tr>
<td>Asymptomatic (n = 8)</td>
<td>1/8</td>
<td>0/8</td>
<td>2/8</td>
<td>6*</td>
</tr>
<tr>
<td>Symptomatic (n = 13)</td>
<td>0/13</td>
<td>4/13</td>
<td>1/13</td>
<td>13*</td>
</tr>
</tbody>
</table>

*Using healthy control samples as the reference group. Fisher exact test: *P < .0001; †P < .01. Nonlabeled occurrences are not significantly different from those of the healthy control.
and 3/23, respectively) compared with healthy control samples (1/40). Logistic regression analysis revealed a significant correlation between HHV-6 infection and the severity of the symptoms (P < .008), i.e., progressing severity of the disease was associated with an increasing frequency of HHV-6 occurrence. Again, a characteristic subtype distribution was observed: HHV-6B infections were significantly associated (P < .01) with symptomatic large-sized lesions (Table I).

We analyzed the overall occurrence of the disease-associated herpesviruses, i.e., our previous EBV and HCMV data were also considered. Altogether, 31 apical periodontitis samples (77%) harbored ≥1 of the tested herpesviruses. Single infection by HHV-6 was observed in 1 apical periodontitis sample harboring subtype A, and the remaining 7 HHV-6 infections were found together with EBV. A similar distribution was found for HCMV: 1 single infection and 3 coinfections were found. Type III latency stage of EBV infection was present in every multiple infected lesion, and 1 lesion harbored triple herpesviral infection. A similar rate of symptomatic manifestation was detected in lesions with single and multiple herpesviral infections (9/22 vs. 5/9; P = .73; Table II).

Four patients provided multiple apical periodontitis samples. Both clinical and virologic analysis of these samples revealed that separate lesions of a patient can differ from each other (Table III). The multiple lesions had different HHV-6 and EBV status in 1 and 2 patients, respectively. Ten individuals from the control group provided multiple samples. Even in the control group, 1 person had one HHV-6A–infected lesion and another lesion uninfected by the investigated herpesviruses.

**DISCUSSION**

Human herpesvirus 6 (HHV-6) infection is known to occur in marginal periodontitis, but no information has been available so far on the presence of this virus in apical periodontitis. In the present study, we determined the PCR prevalence of HHV-6 subtypes A and B in apical periodontitis and analyzed their relationship with previously reported disease-associated herpesviruses EBV and HCMV. The disease association was demonstrated by using healthy pulp as control tissue, which has a histologic continuum through foramen physiologicum to apical periodontium. Like EBV and HCMV, HHV-6 also had a negligible occurrence in the healthy pulp samples of the study. In periapical lesions, the occurrence of HHV-6 exceeded the occurrence of HCMV, a beta-herpesvirus with established disease association. In fact, the prevalence rate of HHV-6 was sufficient to demonstrate a significant association between HHV-6 infection and apical periodontitis. Both beta-herpesviruses tended to occur in coinfection with EBV, which appeared to be the major

| Table II. Single and multiple infections by HHV-6, HCMV, and EBV in apical periodontitis |
|---------------------------------------------|-----------|-----------|-----------|-----------|-----------|
| HHV-6 single infection                      | HCMV single infection | EBV single infection | HHV-6 + EBV coinfection | HCMV + EBV coinfection |
| Healthy control (n = 40)                    | 1          | 0         | 1         | 0         | 0         |
| Apical periodontitis (n = 40)               | 1          | 1         | 20        | 7*        | 3*        |
| Small-sized (<5 mm) (n = 19)                | 1          | 0         | 7         | 2*        | 1*        |
| Asymptomatic (n = 15)                       | 0          | 0         | 8         | 4         | 1         |
| Symptomatic (n = 4)                         | 0          | 0         | 1         | 0         | 0         |
| Large sized (≥5 mm) (n = 21)                | 0          | 0         | 1         | 0         | 0         |
| Asymptomatic (n = 8)                        | 0          | 0         | 4         | 1         | 1         |
| Symptomatic (n = 13)                        | 0          | 0         | 8         | 4         | 1         |

Abbreviations as in Table I.

*One sample with triple infection included.

<p>| Table III. Lesion-related occurrence of EBV, HHV-6, and HCMV in patients providing multiple samples |
|---------------------------------------------------|-----------|-----------|-----------|-----------|-----------|</p>
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sample no.</th>
<th>Tooth position</th>
<th>EBV</th>
<th>HHV-6 subtype</th>
<th>HCMV</th>
<th>Lesion size</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>12</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>≥5 mm</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>22</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>≥5 mm</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>12</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>&lt;5 mm</td>
<td>+</td>
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<td>4</td>
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<td>+</td>
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<td>−</td>
<td>≥5 mm</td>
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<td>19</td>
<td>26</td>
<td>11</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>&lt;5 mm</td>
<td>−</td>
</tr>
<tr>
<td>19</td>
<td>29</td>
<td>12</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>&lt;5 mm</td>
<td>−</td>
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<tr>
<td>20</td>
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<tr>
<td>20</td>
<td>28</td>
<td>13</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>&lt;5 mm</td>
<td>−</td>
</tr>
</tbody>
</table>
viral component in the apical specimens of the present study.

In this study population, we previously found that type III latency EBV infection was present in two-thirds of the EBV-positive apical lesions and was associated with progressive forms of the disease.\textsuperscript{15} It is of note that all EBV/HHV-6 and EBV/HCMV coinfections were found together with type III EBV latency. Therefore, the similar rate of symptomatic manifestation in single and multiple infections was maintained after restricting the analysis to cases with type III EBV latent infection (data not shown). Although HHV-6 infection itself had a significant trend to associate with increasing lesion size and progressing symptoms, further investigations are needed to clarify whether HHV-6 infection is an EBV-dependent or an independent marker of symptomatic manifestation.

Nevertheless, HHV-6 subtypes had a characteristic distribution: Whereas subtype A was found in small or asymptomatic lesions and in a control pulp sample, subtype B was significantly associated with large-sized symptomatic lesions, i.e., the biologic diversity of the 2 HHV-6 subtypes appeared also in apical periodontitis. There were 4 patients with multiple lesions, and each of them provided 2 samples. The results and observations on these lesions revealed that lesions in the same patient could differ in herpesviral infection, lesion size, and symptomatic manifestation. This finding suggests that the local inflammatory environment is the major determinant of herpesviral involvement in apical periodontitis. Even if the host organism is infected by a herpesvirus, the healthy periapical tissues seem to be uninfected. Because the apical periodontitis lesions of separate teeth can develop independently, the characteristics of separate lesions of the same patient can also differ in local herpesviral infection. Therefore, including multiple lesions from few patients could hardly have biased the disease association of the investigated herpesviruses.

Although the presence of herpesvirus-infected mononuclear cells could be considered to be simply a result of immigration into the inflamed area, these cells secrete cytokines with known involvement in the pathogenesis of periapical periodontitis. Our HHV-6 mRNA results indicated a latent stage of infection. Nevertheless, the latent HHV-6 infection can cause phenotypic and functional changes in host cells, through the alteration of cytokine and chemokine signaling. HHV-6 can suppress the secretion of interleukin (IL) 12, which is a critical mediator in TH1 polarized antiviral responses.\textsuperscript{21} HHV-6 infection is also able to suppress interferon-\(\gamma\) and IL-2 production by the host cell and can induce the production of tumor necrosis factor (TNF) \(\alpha\), IL-1\(\beta\), IL-8, and IL-15. In dendritic cells, HHV-6 reduced the expression of major histocompatibility complex class I molecules and the stimulation of allogenic T-cell proliferation.\textsuperscript{22,23} The major receptor molecule for both HHV-6A and B is the ubiquitous CD46 human glycoprotein, which has an important protective effect against autologous complement activation. Because HHV-6 down-regulates the expression of the receptor, it leads to complement-mediated cellular damage in infected tissues.\textsuperscript{24,25}

The coinfection of HHV-6 with EBV is also known in other diseases, such as infectious mononucleosis and Hodgkin disease.\textsuperscript{26,27} Type III latent EBV infection is transient and uncommon in lymphatic tissues of immunocompetent persons. The local environment of inflamed periapical tissues may facilitate the persistence of lymphatic cells with type III EBV latency, which may secrete inflammatory cytokines, i.e., TNF-\(\alpha\), transforming growth factor \(\beta\), and IL-10.\textsuperscript{28} The T\(_{H}1\) suppressive cytokine secretion by EBV and HHV-6 infected cells might promote local escape from immune surveillance in a synergistic way. EBV and HHV-6 infections may contribute to the pathogenesis of periapical flare ups: the cumulative effects of local immunosuppression, immune-mediated tissue destruction and endopatherogenic bacteria may result in increased periapical bone resorption and progressing clinical symptoms.

In conclusion, the present findings revealed that EBV was the most frequent herpesvirus in apical periodontitis, followed by HHV-6 and HCMV. Both beta-herpesviruses tended to occur in coinfection with EBV. A characteristic subtype distribution was also found: HHV-6 subtype B was significantly associated with large-sized and symptomatic periapical lesions. Our findings suggest that EBV and HHV-6B infections can be associated with symptomatic apical periodontitis.

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