Herpesviruses in Endodontic Pathoses: Association of Epstein-Barr Virus with Irreversible Pulpitis and Apical Periodontitis

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Abstract

Irreversible pulpitis and apical periodontitis are inflammatory diseases caused by opportunistic bacteria with possible co-infection with latent herpesviruses. The objectives of this study are to identify herpesviruses, including human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV-1), and Varicella zoster virus (VZV) in patients with irreversible pulpitis ($n = 29$) or apical periodontitis, either primary ($n = 30$) or previously treated ($n = 23$). Using primary and nested polymerase chain reaction (PCR) and reverse transcription-PCR, EBV DNA and RNA were present in endodontic pathoses in significantly higher percentages (43.9% and 25.6%, respectively) compared with healthy pulp controls (0% and 0%, respectively). HCMV DNA and RNA were found in measurable numbers in both endodontic patients (15.9% and 29.3%, respectively) and in healthy pulp controls (42.1% and 10.5%, respectively). HSV-1 DNA was found in low percentages in endodontic patients (13.4%), and only one patient showed the presence of VZV. In conclusion, EBV may be associated with irreversible pulpitis and apical periodontitis. *(J Endod 2009;35:23–29)*

Key Words

Apical cyst, apical granuloma, apical periodontitis, endodontic infections, Epstein-Barr virus, herpes simplex virus, herpesviruses, human cytomegalovirus, irreversible pulpitis, Varicella zoster virus

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Herpesviruses have recently been implicated in the pathogenesis of periapical pathosis, acute periodontitis, and acute inflammation of the gingiva and oral mucosa (1–3). Endodontic disease is a polymicrobial, multistage inflammatory response initiated by the migration of opportunistic microorganisms leading to an influx of inflammatory cells causing pulpitis and periapical periodontitis (1–3). These inflammatory cells may contain herpesviruses, with disease progression moderated by potential reactivation of latent herpesviruses causing additional impairment of host immune response and subsequent acute endodontic inflammation (1–3).

Herpesviruses are composed of three groups: (1) Alphaherpesvirinae (herpes simplex virus group, consisting of herpes simplex virus [HSV] 1 and 2 and Varicella zoster virus [VZV]), (2) Betaherpesvirinae (cytomegalovirus group, consisting of human cytomegalovirus [HCMV] and human herpesviruses 6 and 7), and (3) Gammaherpesvirinae (lymphoproliferative group consisting of Epstein-Barr virus [EBV] and human herpesvirus 8 or Kaposi's sarcoma-associated herpesvirus). HCMV appears to reside in periodontal macrophages and T lymphocytes and latent EBV in periodontal B lymphocytes, and both herpesviruses are often coresidents of endodontic disease. HSV-1 establishes latency in the nerve ganglia. The EBV latent membrane proteins (LMPs) are similar to the receptors of the tumor necrosis factor (TNF) receptor superfamily and can activate immune-signaling pathways to produce several cytokines (4). In addition, LMP-1 variants have been identified in the oral cavity and may have variations in EBV transmission and persistence (5).

Herpesviruses have been identified in endodontic disease and have been proposed to increase the virulence of resident bacterial pathogens by enhancing bacterial adherence and invasiveness into epithelial cells and other mammalian cells (6–8). Destruction of epithelial cells by herpesvirus infection can facilitate the penetration of bacteria into connective tissue. Herpesviruses, including HCMV, inhibit the expression of macrophage surface receptors that recognize lipopolysaccharides (LPS) and components in the gram-negative bacterial cell wall and, thus deter macrophages from destroying invasive gram-negative bacteria. Furthermore, herpesviruses destroy components of the major histocompatibility complex pathway within macrophages and impair the immune defense in its ability to present antigen, silence natural killer cells, stop cell death, and divert antiviral cytokine responses (7, 8). Therefore, herpesvirus infections can promote bacterial colonization and can enhance the growth and pathogenicity of bacteria by disrupting several aspects of the host immune defense.

Although the number of studies examining the role of herpesviruses in oral disease has been increasing, the majority of studies have focused on periodontitis, with no systematic attempt to examine herpesvirus in endodontic patients with varying pulpal and periapical diseases. The objectives of this study were to ascertain the presence of HCMV, EBV, HSV-1, and VZV in endodontic pathoses, including irreversible pulpitis and apical periodontitis, and to determine the potential association of herpesviruses with clinical symptoms, including acute pain and size of radiographic bone destruction. This study analyzed clinical specimens collected from 82 patients exhibiting endodontic disease, including patients with irreversible pulpitis, apical periodontitis, or previously treated with apical periodontitis, and 19 healthy pulp controls. From these specimens, DNA and messenger RNA were isolated and used in polymerase chain reaction (PCR) and reverse transcription (RT)-PCR to determine the presence of herpesviruses.

Materials and Methods

Collection of Endodontic Specimens

Endodontic specimens were collected from patients seeking dental care at the OHSU School of Dentistry. The protocol for collection of extracted teeth and endodontic specimens was reviewed and approved by the OHSU Institutional Review Board. All specimen collections were obtained using informed consent, and patients were anonymously coded for identification. The inclusion criteria for selection of patients were individuals in good health (American Society of Anesthesia I or II) with no severe systemic diseases and who required tooth extraction or endodontic treatment because of the presence of endodontic disease. Most of the patients in this study did not have fever or malaise, with the exception of individuals diagnosed with acute apical abscess. The exclusion criteria were periodontally involved teeth (probing depth is >4 mm, with periodontal bone loss), vertical root fracture, or immature teeth with open apices. All control teeth used for the collection of healthy pulp were third molars or bicuspids with no evidence of caries, restoration, and cracking and no tissue inflammation. All control teeth were extracted for purposes of orthodontic therapy. No follow-up viral screening of individuals examined in this study was conducted. Using clinical data composed of dental history, signs and symptoms, and clinical and radiographic examinations including thermal testing we determined pulpal and periapical diagnoses were determined. The diagnostic terminology was based on the guidelines from the American Board of Endodontics (2007) *Pulpal & Periapical Diagnostic Terminology* and referenced in *Ingle's Endodontics*, 6th edition (9). There were four categories of specimens, divided into subgroups, with 10 to 22 samples within each subgroup. Category 1 consisted of pulp tissues from irreversible pulpitis with subgroup 1 (symptomatic) and subgroup 2 (asymptomatic). Irreversible pulpitis is a "clinical diagnosis based on subjective and objective findings indicating that the vital inflamed pulp is incapable of healing" (9). Symptomatic is in reference to lingering thermal pain, spontaneous pain, and referred pain (9). Asymptomatic is in reference to "no clinical symptoms with the exception of inflammation produced by caries, caries excavation or trauma" (9). Category 2 consisted of periapical tissues from primary apical periodontitis, with subgroup 1 (symptomatic, including all acute and chronic apical abscesses) and subgroup 2 (asymptomatic or no clinical symptoms with the exception of apical inflammation shown as periapical radiolucent lesions). Category 3 consisted of periapical tissues from previously treated with apical periodontitis, with subgroup 1 (symptomatic, including all acute and chronic apical abscesses) and subgroup 2 (asymptomatic or no clinical symptoms with the exception of apical inflammation shown as periapical radiolucent lesions). In category 4, healthy pulp tissues from third molars or bicuspids were used as controls.

The pulpal and periapical tissue specimens were collected during endodontic treatment or after tooth extraction, with minimal risk of salivary contamination. For pulpal specimens, after rubber dam isolation and caries/restoration removal and before penetration into the pulp, the access was disinfected with 5.25% NaOCl and deactivated with sterile 5% sodium thiosulphate, and then pulpal tissue was subsequently extirpated. For extracted teeth, after surface disinfection with 5.25% NaOCl and deactivation with 5% sodium thiosulphate, the roots were split after vertical grooves had been cut around the roots, and pulp tissue was collected. For the periapical tissue specimens from the extracted tooth, the attached periapical tissue was removed from the root apexes with a sterile blade. Specimens from previously treated teeth with apical periodontitis were collected during the apical surgery. Briefly, before administrating local anesthetics, patients rinsed with 0.12% chlorhexidine mouthwash for 1 minute. Using a sterile no. 15 blade, incisions were extended 1 to 2 teeth mesially and distally from the study tooth followed by a vertical releasing incision. A full-thickness mucoperiosteal flap was then reflected, and the periapical lesion was exposed with a sterile round burr using saline as coolant. A sterile curette was used to obtain the periapical specimen. After harvesting, the specimens were cut into portions, one stored at -85°C immediately for genomic DNA isolation and the other submerged into RNA*later* RNA Stabilization Reagent (Qiagen, Inc, Valencia, CA) and then stored at -85°C for messenger RNA isolation. For most of the previously treated with apical periodontitis cases, a portion of the specimen was fixed in 10% formalin, and histological examinations were conducted by the Oral Pathology Laboratory at the OHSU School of Dentistry.

Extraction of Nucleic Acids from Endodontic Specimens

The tissue specimens were minced with sterile scalpels, and commercial DNA and RNA extraction kits were used. For genomic DNA isolation, the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) was used; this isolation procedure is based on proteinase K extraction and binding and elution of nucleic acids on spin columns using chaotropic salts. For RNA isolation, the RNeasy Mini Kits (Qiagen, Inc) were used; extracted samples were subjected to RNase-free DNase I digestion (10 units, 37°C, 30 minutes) before processing and completion of RNA isolation.

PCR, RT-PCR, and Nested PCR

SuperScript III CellsDirect cDNA Synthesis System (Invitrogen) was used for first-strand complementary DNA synthesis using random hexamer primers. Using control recombinant or viral genomic DNA templates (see below), PCR conditions were optimized, and selected polymerases included Platinum *Taq* DNA High Fidelity (Invitrogen) for HCMV and HSV-1 amplifications, the Pfx50 DNA Polymerase (Invitrogen) for EBV amplifications, and Go *Taq* Green Master Mix (Promega, Madison, WI) for VZV amplifications. The amplification of human beta actin sequences in RT-PCR was used to monitor the fidelity of the complementary DNA synthesis. PCR was conducted with an initial denaturation step at 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes followed by a 4°C soak. Nested PCR was used to amplify the low herpesvirus copy number found in the endodontic specimens and to increase the sensitivity of PCR fragment detection.

Primers, Control Recombinant and Genomic DNA from Cell Lines, and DNA Sequencing

Primers used in PCR and nested PCR for amplification of HCMV, EBV, HSV-1, and VZV sequences are displayed in Table 1. Primers were designed to amplify sequences from the HCMV pp65 lower matrix phosphoprotein (UL 83 gene; accession number NC 001347), the EBV BLR F2 gene product (EBV structural protein, potentially a tegument protein); EBV type 1, accession number NC 007605; EBV type 2, NC 009334), HSV-1 RL2 immediate early gene product (latency; accession number NC 001806), and VZV ORF 29 gene product (gp31; single-stranded DNA-binding protein involved in DNA replication; accession number NC 001348). DNA controls include (1) recombinant plasmids containing HCMV pp65, HSV-1, and human beta actin sequences (provided by Mark Stinski, University of Iowa, Iowa City, IA; Patricia Spear, Northwestern University, Chicago, IL; Richard Longnecker, Northwestern University, Chicago, IL), (2) genomic DNA from HCMV-transformed lymphoid cells (provided by Sunwen Chou, Department of Veterans Affairs Medical Center, Portland, OR), (3) genomic DNA and cell pellets (for RNA extractions) of EBV-transformed lymphoblastoid cell lines and matching nontransformed controls (obtained from American Type Cul-

TABLE 1. Primer Sequences of HCMV, HSV, EBV, VZV and Actin

Target molecule	Accession number	Primer description	Primer location*	Primer sequence	Size of amplicon
HCMV†	NC_001347	Primary upstream	121314→121333	5′ TCACCTGCATCTTGGTTGCG 3′	
		Primary downstream	121622→121603	5′ TGCCGCTCAAGATGCTGAAC 3′	309 bp
		Nested upstream	121403→121422	5′ GGAAACACGAACGCTGACGT 3′	
		Nested downstream	121622→121603	5′ TGCCGCTCAAGATGCTGAAC 3′	220 bp
EBV type 1‡	NC_007605	Primary upstream	76641→76661	5′ CAGCTCCACGCAAAGTCAGAT 3′	
		Primary downstream	77122→77101	5′ ATCAGAAATTTGCACTTTCTTT 3′	482 bp
		Nested upstream	76680→76699	5′ TTGACATGAGCATGGAAGAC 3′	
		Nested downstream	77042→77022	5′ CTCGTGGTCGTGTTCCCTCAC 3′	363 bp
EBV type 2‡	NC_009334	Primary upstream	76775→76795	5′ CAGCTCCACGCAAAGTCAGAT 3′	
		Primary downstream	77256→77235	5′ ATCAGAAATTTGCACTTTCTTT 3′	482 bp
		Nested upstream	76814→76833	5′ TTGACATGAGCATGGAAGAC 3′	
		Nested downstream	77176→77156	5′ CTCGTGGTCGTGTTCCCTCAC 3′	363 bp
HSV-1	NC_001806	Primary upstream	120665→120674	5′ CCAACACAGACAGGGAAAAG 3′	
		Primary downstream	121000→120981	5′ GGAACATGCTGTTCGACCAG 3′	336 bp
		Nested upstream	120703→120723	5′ AGACAGCAAAAATCCCCTGAG 3′	
		Nested downstream	120898→120880	5′ ACGAGGGAAAACAATAAGG 3′	196 bp
VZV	NC_001348	Primary upstream	51067→51089	5′ ACGGGTCTTGCCGGAGCTGGTAT 3′	
		Primary downstream	51338→51315	5′ AATGCCGTGACCACCAAGTATAAT 3′	272 bp
		Nested upstream	51099→51118	5′ ACTCACTACCAGTCATTTCT 3′	
		Nested downstream	51306→51286	5′ TTCTGGCTCTAATCCAAGGCG 3′	208 bp
Human β-actin	NM_00101	Upstream	1141→1162	5′ CAGCAGATGTGGATCAGCAAGC 3′	
		Downstream	1506→1485	5′ AGGATGGCAAGGGACTTCCTGT 3′	366 bp

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, Varicella zoster virus.

*Primer locations are relative to the positions in the complete viral genomic sequence referenced in individual accession numbers. Herpesviral genomic sequences and human beta actin sequences were available for download from the US National Library of Medicine (www.pubmed.gov).

†HCMV primary downstream and nested downstream primers are identical.

‡Primary and nested primers for EBV type 1 and type 2 are conserved for both EBV types. Thus, our EBV PCR primers simultaneously recognize and amplify fragments for both EBV type 1 and type 2.

ture Collection, Manassas, VA), (4) genomic DNA from the EBV-containing Ramos, Namalwa, and Raji cell lines (provided by Astrid Meerbach, Institute of Virology and Antiviral Therapy, Friedrich-Schiller University Jena, Germany), and (5) VZV genomic DNA from Randall Cohrs (University of Colorado Health Science Center, Denver, CO). PCR products were electrophoresed in 1.2% agarose gels containing ethidium bromide (0.5 μg/mL) and visualized using transillumination at 320 nm. PCR products were sequenced using an ABI 3130xl DNA analyzer at the Molecular Microbiology and Immunology Research Core Facility, OHSU School of Medicine.

Statistical Analysis

Chi-square analysis and a Fisher exact test were conducted to assess statistical significance between categoric data. Dr Hong Li in the Department of Public Health and Preventative Medicine, OHSU School of Medicine, conducted the statistical analysis using the SAS software, a business intelligence and predictive analytics software developed by SAS Institute Inc. (Cary, NC).

Results

Presence of Herpesviruses in Irreversible Pulpitis and Apical Periodontitis

A total of 101 specimens were collected from patients (age range, 15–92 years; average age, 41.5 years; 50 males and 51 females). Specimens were obtained from molars (62), bicuspids (20), and anterior teeth (19). Table 2 shows the incidence of herpesviruses in endodontic pathoses. EBV DNA was present in patients with irreversible pulpitis

TABLE 2. Incidence of Herpesviruses in Endodontic Pathoses

Endodontic pathoses	N	HCMV		EBV		HSV		VZV	
		DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA
Healthy pulp	19	8 (42.1)	2 (10.5)	0	0	1 (5.26)	0	0	0
Irreversible pulpitis	29	8 (27.6)	7 (24.1)	9 (31.0)†	2 (6.90)	6 (20.7)	0	1 (3.45)	1 (3.45)
Subgroup 1: symptomatic	19	5 (26.3)	4 (21.1)	5 (26.3)*	0	3 (15.8)	0	1 (5.26)	1 (5.26)
Subgroup 2: asymptomatic	10	3 (30)	3 (30)	4 (40)†	2 (20)	3 (30)	0	0	0
Apical periodontitis	30	4 (13.3)	11 (36.7)	16 (53.3)†	13 (43.3)†	2 (6.67)	0	0	0
Subgroup 1: symptomatic	18	2 (11.1)	5 (27.8)	9 (50)†	6 (33.3)†	2 (11.1)	0	0	0
Subgroup 2: asymptomatic	12	2 (16.7)	6 (50)	7 (58.3)†	7 (58.3)†	0	0	0	0
Previously treated with apical periodontitis	23	1 (4.35)	6 (26.1)	11 (47.8)†	6 (26.1)*	3 (13.0)	0	0	0
Subgroup 1: symptomatic	14	1 (7.14)	5 (35.7)	8 (57.1)†	5 (35.7)†	3 (21.4)	0	0	0
Subgroup 2: asymptomatic	9	0	1 (11.1)	3 (33.3)†	1 (11.1)	0	0	0	0
Total	82	13 (15.9)*	24 (29.3)	36 (43.9)†	21 (25.6)†	11 (13.4)	0	1 (1.22)	1 (1.22)

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, Varicella zoster virus.

Values are given as n (%).

*$p < 0.05$.

†$p < 0.01$.

TABLE 3. Incidence of Herpesviruses in Symptomatic versus Asymptomatic Endodontic Pathoses

	N	HCMV		EBV		HSV		VZV	
		DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA
Total									
Symptomatic	51	8 (15.7)	14 (27.5)	22 (43.1)	11 (21.6)	8 (15.7)	0	1 (1.96)	1 (1.96)
Asymptomatic	31	5 (16.1)	10 (32.3)	14 (45.2)	10 (32.3)	3 (9.68)	0	0	0

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, Varicella zoster virus.
Values are given as n (%).

(9/29), primary apical periodontitis (16/30), and previously treated with apical periodontitis (11/23), all in high percentages (31.0%, 53.3% and 47.8%, respectively) compared with incidence in healthy pulp controls (0/19, all comparisons have $p < 0.05$; Table 2). EBV RNA was actively transcribed in patients with irreversible pulpitis or apical periodontitis, either primary or previously treated (6.9%, 43.3% and 26.1%, respectively), in higher percentages compared with healthy pulp controls (0%, all comparisons have $p < 0.05$, Table 2). All cases had statistically significant higher incidence of EBV DNA and RNA in endodontic pathoses versus healthy pulp controls ($p < 0.05$, Table 2). Interestingly, while HCMV DNA was found in measurable numbers in patients with irreversible pulpitis and in apical periodontitis both primary or previously treated (27.6%, 13.3% and 4.4%, respectively), HCMV DNA was also found in high percentages in healthy pulp controls (42.2% of specimens surveyed). In addition, HCMV RNA was found at even higher overall frequencies in irreversible pulpitis and in apical periodontitis both primary or previously treated (24.1%, 36.7% and 26.1%, respectively), compared to 10.5% for healthy pulp controls (Table 2), but these differences were not determined to be statistically significant. In the irreversible pulpitis group, 7 out of 29 patients (24.1%) contained both EBV and HCMV. In the primary apical periodontitis group, 11 out of 30 patients (36.7%) contained both EBV and HCMV, and in the previously treated apical periodontitis group, six out of 23 patients (26.1%) contained both EBV and HCMV (data not shown). HSV-1 DNA was found in slightly higher percentages in irreversible pulpitis and in apical periodontitis both primary and previously treated (20.7%, 6.7% and 13.0%, respectively) compared with the incidence of HSV-1 in healthy pulp controls (5.3%), but these differences were also not statistically significant. No HSV-1 RNA was found in any of the diseased or healthy pulp specimens. Only one patient, exhibiting symptomatic irreversible pulpitis, showed presence of VZV DNA and RNA. In all positive identifications confirming the presence of EBV, HCMV, HSV-1, and VZV in the endodontic specimens, the electrophoretic sizes of the PCR products corresponded exactly with the predicted sizes based on sequence and matched the sizes of the PCR products generated by recombinant or genomic viral DNA controls. All fragments were also directly verified by sequence analysis. Fragment sequences were aligned with herpesviral genomic DNA sequences obtained from the National Library of Medicine/National Institutes of Health (www.pubmed.gov). The EBV primers were designed to recognize identical target sequences between EBV types 1 and 2 and generated identically sized PCR fragments; in addition, the 363-bp fragments amplified during nested PCR were nearly identical in sequence between the two EBV strains. However, the fragment sequence had one mismatch compared with EBV type 1 sequence (362/363 matches or 99.7% identity) versus four mismatches compared with EBV type 2 sequence (359/363 matches or 99.2% identity). Although the sequence of the amplified region obtained from EBV type 1 and EBV type 2 are too similar to effectively distinguish the two EBV strains, it appears more likely that the endodontic specimens may have contained EBV type 1.

Presence of Herpesviruses in Symptomatic versus Asymptomatic Endodontic Pathoses

The patients were grouped according to symptomatic or asymptomatic classifications (Table 3). Statistical analyses revealed that there were no significant differences in the incidence of HCMV, EBV, HSV-1, or VZV between the symptomatic ($n = 51$) versus asymptomatic ($n = 31$) groups for both DNA and RNA analyses.

Presence of Herpesviruses in Apical Periodontitis Subdivided According to Size of Radiographic Bone Destruction

The patients were grouped according to the size of radiographic bone destruction ($\geq$5 mm or $<$5 mm, Table 4) in both primary and previously treated with apical periodontitis. Thirty-six patients had radiographic bone destruction of $\geq$5 mm, and 17 patients had radiographic bone destruction of $<$5 mm. From the patients with radiographic bone destruction $\geq$5 mm, HCMV, EBV, HSV-1, and VZV DNA were found in 13.9%, 50%, 13.9%, and 0% of the patients, respectively, and HCMV, EBV, HSV-1, and VZV RNA were found in 33.3%, 36.1%, 0%, and 0% of the patients, respectively. From the patients with radiographic bone destruction $<$5 mm, 47.1% of the patients contained EBV DNA, whereas none of the patients contained HCMV, HSV-1, or VZV DNA. In

TABLE 4. Radiographic Lesion Size and Incidence of Herpesviruses in Apical Periodontitis

Apical periodontitis lesion size	N	HCMV		EBV		HSV		VZV	
		DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA
Primary apical periodontitis									
> 5 mm	18	4 (22.2)	6 (33.3)	10 (55.6)	9 (50)	2 (11.1)	0	0	0
< 5 mm	12	0	5 (41.7)	5 (41.7)	4 (33.3)	0	0	0	0
Previously treated with apical periodontitis									
> 5 mm	18	1 (5.55)	6 (33.3)	8 (44.4)	4 (22.2)	3 (16.7)	0	0	0
< 5 mm	5	0	0	3 (60)	2 (40)	0	0	0	0
Total									
> 5 mm	36	5 (13.9)	12 (33.3)	18 (50)	13 (36.1)	5 (13.9)	0	0	0
< 5 mm	17	0	5 (29.4)	8 (47.1)	6 (35.3)	0	0	0	0

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, Varicella zoster virus.
Values are given as n (%).

TABLE 5. Histological Classification and Incidence of Herpesviruses in Specimens with Previously Treated Apical Periodontitis

Histological classification	N	HCMV		EBV		HSV		VZV	
		DNA	mRNA	DNA	mRNA	DNA	mRNA	DNA	mRNA
Apical cyst	6	0	2 (33.3)	2 (33.3)	0	2 (33.3)	0	0	0
Apical granuloma	11	1 (9.09)	4 (36.4)	6 (54.6)	4 (36.4)	1 (9.09)	0	0	0
Subgroup 1: with acute inflammation	4	0	3 (75)	3 (75)	1 (25)	0	0	0	0
Subgroup 2: without acute inflammation	7	1 (14.3)	1 (14.3)	3 (42.9)	3 (42.9)	0	0	0	0
Total	17	1 (5.88)	6 (35.3)	8 (47.1)	4 (23.5)	3 (17.7)	0	0	0

HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, Varicella zoster virus.
Values are given as *n* (%).

addition, from patients with radiographic bone destruction <5 mm, 29.4% and 35.3% of the patients contained HCMV or EBV RNA, respectively, whereas none of the patients contained HSV-1 or VZV RNA. Thus, there were no significant differences in the incidence of HCMV, EBV, HSV-1, or VZV between groups containing small radiographic lesions (<5 mm) versus large radiographic lesions (≥5 mm).

Presence of Herpesviruses in Apical Periodontitis Subdivided into Histological Classifications

Histopathological examinations were conducted on 17 specimens from the previously treated with apical periodontitis group. Among the 17 specimens, 6 were found to be apical cysts and 11 were found to be apical granulomas (Table 5). Out of the 11 patients with apical granulomas, 4 and 7 patients presented with or without acute inflammation, respectively. HCMV, EBV, HSV-1 and VZV DNA were found in 1, 8, 3, and 0 patients, respectively. HCMV, EBV, HSV-1, and VZV RNA were found in 6, 3, 0, and 0 patients, respectively. Thus, there were no statistically significant differences in the incidence of herpesviruses between groups consisting of apical cysts versus apical granulomas, either with or without inflammation (Table 5).

Discussion

Overview of Herpesviruses in Endodontic Pathoses

Herpesviruses have been implicated in the pathoses of symptomatic and asymptomatic apical periodontitis lesions (1–3). Using RT-PCR, Sabeti et al (1) found that most teeth with necrotic pulp and periapical lesions harbored herpesvirus in periapical granulomatous tissues and that herpesviruses were detected in large symptomatic periapical lesions at a higher incidence when compared with small asymptomatic periapical lesions. Yildirim et al (6) have shown that herpesviruses and bone resorption-inducing cytokines were present in periapical lesions of deciduous teeth. Using an immunohistochemical approach, Saboia-Dantas et al (10) have found HCMV and EBV in apical periodontitis lesions, with higher prevalence in HIV-positive patients. Using PCR, Andric et al (11) detected HCMV in the cystic wall and determined that HCMV were contained in both inflammatory and noninflammatory odontogenic cysts. This current study analyzed clinical specimens from 82 patients exhibiting endodontic disease, including patients with irreversible pulpitis and primary or previously treated with apical periodontitis and 19 healthy pulp controls, and used PCR and RT-PCR to determine the presence of herpesviruses and association with pupal and periapical diagnoses, including endodontic disease classification, presence of symptoms, size of radiographic bone destruction, and histological classifications.

EBV and Association with Endodontic Pathoses

Both EBV DNA and RNA were present in endodontic disease specimens (in irreversible pulpitis, primary apical periodontitis, and previously treated with apical periodontitis) at significantly higher levels than in normal pulp specimens ($p = 0.007$, $p < 0.0001$, and $p = 0.0003$, respectively; Table 2). When examined by using even more specific endodontic disease classifications, including symptomatic or asymptomatic irreversible pulpitis, symptomatic or asymptomatic primary apical periodontitis, and symptomatic or asymptomatic previously treated with apical periodontitis, all individual comparisons were significantly higher in numbers of patients containing EBV DNA when compared with healthy pulp specimens ($p = 0.046$, $p = 0.009$, $p = 0.0004$, $p = 0.0003$, $p = 0.0002$, and $p = 0.026$, respectively). Interestingly, when examining the EBV RNA data, there were significantly higher numbers of patients containing EBV RNA in the primary and previously treated with apical periodontitis disease categories compared with healthy pulp controls ($p = 0.0006$ and $p = 0.0244$, respectively) but not in the case of the comparison of irreversible pulpitis ($p = 0.512$) with control pulp.

Lack of Association of HCMV, HSV-1, or VZV with Endodontic Pathoses and Other Diagnostic Indicators

When similar comparisons were made between the numbers of patients containing HCMV DNA or RNA, HSV-1 DNA, or VZV DNA or RNA in irreversible pulpitis or apical periodontitis compared with healthy pulp, no statistical differences were found, indicating there was no apparent association of HCMV, HSV-1, or VZV to these endodontic disease categories (Table 2). The lack of association of HSV-1 to endodontic pathoses is consistent with the findings of Sabeti et al (1). Interestingly, a case study linking VZV with the identification of a patient with symptoms mimicking irreversible pulpitis may have been the result of misdiagnosis with herpes zoster (12) and is supportive of the premise that VZV is not associated with endodontic pathosis. EBV DNA and RNA are found in near equivalent frequencies in symptomatic and asymptomatic endodontic pathoses (Table 3), indicating that the presence of EBV is not a predictor for the incidence of pain. When examining radiographic bone destruction and focusing on EBV, there appears to be no significant difference in the distribution of EBV DNA or RNA in radiographic lesions ≥5 mm versus radiographic lesions <5 mm (Table 4). Furthermore, when examining the presence of herpesviruses in patients previously treated with apical periodontitis, there appears to be no significant differences in the distribution of these viruses between apical cysts or apical granuloma (Table 5).

Endodontic Pathoses and Other Considerations

This investigation examined the incidence of herpesviruses in both irreversible pulpitis and apical periodontitis, with only the latter disease being investigated in previous studies. Thus, this current study and conclusion that EBV is associated with irreversible pulpitis represents the first analysis for this endodontic pathosis. An investigation conducted by Sabeti et al (1) identified almost 100% incidence of HCMV in

specimens from patients previously treated with apical periodontitis, with HCMV RNA being present in considerable quantities for detection by primary PCR. Furthermore, Yildirim et al (6) examined periapical specimens from deciduous teeth, believed to be similar to the specimens collected from patients presenting with symptomatic apical periodontitis in this current study; Yildirim et al (6) found HCMV and EBV DNA in 58% and 67% of the specimens. These results are in contrast to this current study in which much lower frequencies of HCMV (4.35% of patients with HCMV DNA and 26.1% of patients with HCMV RNA) are found in patients previously treated with apical periodontitis. Also, in this current study, HCMV DNA was present in much lower copy number that required nested PCR for detection. In addition, contrary to Sabeti et al (1), HCMV was found in healthy pulp specimens at frequencies (42.1% of specimens with HCMV DNA and 10.5% of specimens with HCMV RNA) that were statistically equivalent to those found in endodontic pathoses specimens. Results from this current study are more consistent with the findings of Saboia-Dantas et al (10) and Sunde et al (13). Saboia-Dantas et al (10) have identified HCMV and EBV in 23% and 31% of patients with apical periodontitis, respectively, and Sunde et al (13) have found EBV to be the predominant herpesvirus in apical periodontitis (50% incidence) compared with HCMV, which was not detected in their specimens.

EBV Latency and Immune Impairment and Effects on Endodontic Pathoses

EBV is an important human pathogen that is contained in greater than 90% of the world population and is associated with several diseases, including infectious mononucleosis (14, 15), malignant lymphomas, and nasopharyngeal carcinoma (16–19). EBV transmission occurs via infected saliva or blood, resulting in the establishment of latent or persistent infection of B lymphocytes and chronic asymptomatic shedding of virus particles (14, 15). Latent EBV infection results in the restricted expression of viral genes and therefore escapes immune surveillance (14, 15). EBV can replicate in oral epithelial cells but may also be present in oral tissues in a nonproductive or latent state (16–19). It is believed that prolonged periods of latency and periods of reactivation may be partially responsible for the symptomatic and asymptomatic transitioning observed for apical periodontitis (10). Nested PCR experiments, which detect herpesviral DNA, are unable to distinguish latent episomal viral DNA versus genomic viral DNA contained in virus particles that would be indicative of an active infection. This may account for some of the differences observed between frequencies of herpesvirus DNA (from either a latent or active infection) versus herpesvirus RNA (transcription indicative of an active infection) in patients with specific endodontic pathoses. In addition, the EBV primers were designed to recognize identical target sequences and generate identically sized PCR fragments from the BLR F2 gene of EBV types 1 and 2 (Table 1), with near identical sequence for the entire amplified region. Although the sequence of the amplified region obtained from EBV type 1 and EBV type 2 are too similar to effectively distinguish the two EBV strains, it appears more likely that the endodontic specimens may have contained EBV type 1. The *BLR F2* gene is believed to encode EBV structural proteins, potentially tegument proteins, and would be transcribed during an active EBV infection. Thus, detection of EBV RNA, as observed in 21 out of 82 patients (25.6%) with endodontic pathoses, supports the premise that an active EBV infection had occurred with the generation and release of virus particles. In addition, EBV types 1 and 2 contain sequence divergence in the EBV nuclear antigen 2 (EBNA2) and EBNA3 genes (4, 5, 16). The profile of EBV strains and patterns of abundance in the oral cavity and blood provide evidence for compartmentalization of specific EBV strains and potential progression of disease (17). It may be possible that specific EBV strains are involved in the symptomatic or asymptomatic forms of apical periodontitis, and further study is required to distinguish the importance of different EBV strains in endodontic pathoses.

It should be noted that the recurrence of apical periodontitis may be caused by other cofactors, including bacteria, which may survive because of the virus-induced weakening of the immune system to clear microbial invaders. EBV DNA and RNA are found in many but not all patients with irreversible pulpitis and apical periodontitis. Although statistically significant in supporting the premise that the presence of EBV is associated with endodontic pathoses, the fact that it is not found in all endodontic patients allows the interpretation that other microorganisms may serve as cofactors of disease. Furthermore, herpesvirus-infected immune cells may release cytokines that could lead to inflammation and exacerbation of symptoms including pain.

In conclusion, our results have indicated that EBV is present in significantly higher frequencies in irreversible pulpitis and apical periodontitis when compared with healthy controls. Furthermore, this current study supports the premise that EBV, but not HCMV, HSV-1, or VZV, is associated with irreversible pulpitis and apical periodontitis and may potentially play a role in endodontic disease.

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