

Significance of Human Cytomegalovirus and Epstein-Barr Virus in Inducing Cytokine Expression in Periapical Lesions

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Abstract

Introduction: Because herpesviruses might be etiologically involved in periapical pathosis of endodontic origin, this study aimed to determine the occurrence of human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and the expression of mRNA transcripts of tumor necrosis factor (TNF)- α , γ -interferon (IFN), interleukin (IL)-1 β , IL-6, IL-12, and IL-10 in periapical granulomatous lesions collected in conjunction with apicoectomy.

Methods: A total of 9 symptomatic and 6 asymptomatic teeth with periapical lesions were studied. Periapical samples were collected in conjunction with apicoectomy, which was being performed because of radiographic evidence of incomplete periapical healing after conventional root canal therapy. By using established polymerase chain reaction primers and procedures, polymerase chain reaction assays were used to identify herpesvirus and cytokine gene expression.

Results: The difference in occurrence of HCMV, EBV, and cytokines between symptomatic and asymptomatic periapical lesions was statistically significant: HCMV ($P = .048$), EBV ($P = .002$), IFN ($P = .001$), IL-1 ($P = .012$), IL-6 ($P = .026$), IL-10 ($P = .026$), IL-12 ($P = .012$), and TNF ($P < .001$) (Mann-Whitney U test). There was a significant correlation between EBV, HCMV, and TNF, γ -IFN, IL-1, and IL-12 in symptomatic periapical lesions (Spearman test).

Conclusions: The present findings provide evidence of a putative role of HCMV and EBV in the pathogenesis of symptomatic periapical pathosis. The release of tissue-destructive cytokines might be of pathogenetic significance. (*J Endod* 2012;38:47–50)

Key Words

Cytokine, herpesvirus, PCR, periapical lesion

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Periapical lesions are infectious disorders that exhibit a variety of clinical and radiographic manifestations. It remains to be determined whether different clinical signs and/or symptoms and histopathologic classification of periapical lesions have distinct microbial causation, or whether they arise from varying host immune reactions to infectious agents.

Recent studies have investigated the occurrence of herpesviruses in periapical granulomas (1–4). cDNA identification of genes transcribed late during the infectious cycle of herpesviruses was used to indicate herpesvirus active infection (1). The findings obtained revealed a strong association of human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) with symptomatic periapical pathosis. Periapical infection, harboring CMV-EBV dual infection, tended to show elevated occurrence of anaerobic bacteria, to be symptomatic, and exhibited large-size radiographic bone destruction (4). HCMV and EBV active infection leads to activation of mammalian cells and production of numerous cytokines and chemokines, which can induce further cell activation and cytokine production in a complex system of regulation and cross-regulation (5). Cytokines have been implicated in the development, maintenance, and healing of periapical lesions (6).

An important research question is to determine the extent to which periapical herpesviruses influence the release of tissue-destructive cytokines and the progressive course of various types of endodontic disease. Information is still sparse on herpesviruses in periapical symptomatic lesions, and the cytokine production in such lesions needs to be further elucidated. Therefore, this study examined the presence of HCMV and EBV DNA and the expression of mRNA transcripts of tumor necrosis factor (TNF)- α , γ -interferon (IFN), interleukin (IL)-1 β , IL-6, IL-12, and IL-10 in periapical granulomatous lesions collected in conjunction with apicoectomy. The present study also investigated a possible relationship of periapical HCMV and EBV with the cytokines studied. Polymerase chain reaction (PCR) assays were used to identify the cytokine molecules.

Materials and Methods

A total of 9 symptomatic and 6 asymptomatic teeth with periapical lesions were studied. Symptomatic teeth exhibited swelling, pain, discomfort on biting, or sensitivity by percussion or palpation. Asymptomatic teeth revealed no previous signs or symptoms of acute periapical disease. None of the studied teeth demonstrated moderate or severe types of marginal periodontitis.

Periapical samples were collected in conjunction with apicoectomy, which was performed because of radiographic evidence of incomplete periapical healing after conventional root canal therapy. Before administering local anesthetics, the teeth, gingiva, and mucosa of the sample area were washed with 0.12% chlorhexidine; patients rinsed with 0.12% chlorhexidine mouthwash for 30 seconds. By using a sterile no. 15 blade, an intrasulcular incision was extended 1–2 teeth mesially and distally from the study tooth. This was followed by a vertical release incision mesially. A full-thickness mucoperiosteal flap was then reflected, exposing the periapical lesion area. Access through the cortical bony plate was obtained with a sterile explorer and/or no. 4 or 6 sterile high-speed surgical round burs, with sterile water coolant in the area of osteotomy. By using a sterile curette, a periapical specimen for virologic identification was placed in an empty plastic vial and immediately frozen.

Samples for PCR assays, which were obtained immediately after apicoectomy, were placed in 500 μ L denaturing solution of the EZ-RNA total RNA isolation kit (Biological

TABLE 1. PCR Primers Used in the Study

Target molecule	PCR primer	Size of amplicon (base pairs)
HCMV		
Forward primer	5'-ACGCGCTGCCGCTCAAGAT-3'	207
Reverse primer	5'-TGTAGTAGACGTCGGGCTCTTT-3'	
EBV		
Forward primer	5'-TCCACCACCCAGGCAC-3'	197
Reverse primer	5'-TGGAGAGGTCAGGTTACTTAC-3'	
TNF- α		
Forward primer	5'-CTTCAAGGGCCAAGGCTGC-3'	180
Reverse primer	5'-CCAGATAGATGGGCTCATACCA-3'	
γ -IFN		
Forward primer	5'-TAATGCAGAGCCAAATTGTCTCC-3'	189
Reverse primer	5'-GCGTTGGACATTCAAGTCAGTTA-3'	
IL-1B		
Forward primer	5'-CGACACATGGGATAAGAG-3'	200
Reverse primer	5'-CCAAGGCCACAGGTATTTTG-3'	
IL-6		
Forward primer	5'-GGTACATCCTCGACGGCATCT-3'	194
Reverse primer	5'-GT GCCTCTTTGCTGCTTTCAC-3'	
IL-10		
Forward primer	5'-CTTCGAGATCTCTCCGAGATGC-3'	260
Reverse primer	5'-AAGAAATCGATGACAAGCGCCG-3'	
IL-12		
Forward primer	5'-GCCACAGGTCTGCATCCA3'	106
Reverse primer	5'-GACCTGGCGGGCTGAGTA-3'	

Industries, Kibbutz Beit-Haemek, Israel). The tissue samples were then homogenized with a low-rotary device and divided into 2 aliquots. One aliquot was used for the extraction of viral DNA and one aliquot for total RNA isolation. Extracted DNA or RNA was resuspended in 100 μ L of distilled water, and 5 μ L of the DNA or RNA solution was used in the PCR assay.

The primers were designed and analyzed by using Oligoware 1.0 software program (7) and synthesized by MWG Biotech (Ebersberg bei München, Germany). PCR for detecting herpesviruses was performed with a final volume of 50- μ L mixture containing 30 pmol of each primer (MWG-Biotech), 2 μ L *Taq* DNA polymerase (Bioron, Ludwigshafen, Germany), 1.5 mmol/L $MgCl_2$, 0.1 mmol/L deoxyribonucleoside triphosphate mix, 5 μ L of 10 \times Reaction Buffer (Bioron), and 5 μ L of extracted DNA sample. PCR procedures included a 40-round amplification process and were performed in 3 steps covering denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 40 seconds. Positive and negative controls for HCMV and EBV included infected and noninfected leukocytes from human peripheral blood.

By using the EZ-RNA total RNA isolation kit according to the package insert instructions (Biological Industries), total RNA for detecting cytokine gene transcripts was prepared from the homogenized tissue samples. Extracted RNA was dissolved in 100 μ L of DNase-free and RNase-free distilled water.

Reverse transcriptase (RT)-PCR reaction was performed in 50 μ L of RT mix (10 U RT [Fermentase, Vilnius, Lithuania], 0.15 mmol/L deoxyribonucleoside triphosphate mix, and 10 μ L of 5 \times RT Buffer [Fermentase]). After incubation at 42°C, PCR procedures, which included a 40-round amplification process, were performed in 3 steps covering (1) denaturation at 94°C for 30 seconds, (2) annealing at 57°C (59°C) for 30 seconds, and (3) extension at 72°C for 40 seconds. Each set of PCR analysis included a negative control (water blank) and a positive control (cloned plasmids). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also served as an internal control.

The specificity of the PCR procedures was confirmed by examining the size of the amplicons and the restriction endonuclease digest of the amplicons. The PCR detection limit, determined by using serially diluted

(10¹–10⁴) cloned plasmid DNA for herpesviruses and cytokines, was found to be 100 cloned plasmids/mL.

Detection of PCR amplicon products was performed electrophoretically in 2% agarose gel containing 0.5 μ g/mL ethidium bromide. Each test run included a size marker (100 base pair PCR marker; Bioron). The intensity of the amplicon band was compared by Quantity One software (BioRad Laboratories, Hercules, CA).

Results

The Mann-Whitney *U* test was used to compare the PCR results from symptomatic and asymptomatic periapical tissue samples. Spearman test was used to assess the relationship between herpesvirus presence and cytokine expression in the periapical tissue samples.

Table 1 illustrates PCR primers used in this study. Tables 2 and 3 display quantification of HCMV, EBV, and various cytokine expressions by real-time RT-PCR in symptomatic and asymptomatic periapical lesions. Table 2 demonstrates the presence of HCMV in 5 of 9 (55%) and EBV in 8 of 9 (88.9%) symptomatic periapical lesions. The expression of cytokines, HCMV, and EBV coinfection was detected in 4 of 9 of the symptomatic lesions. The difference in occurrence of HCMV, EBV, and cytokines between symptomatic and asymptomatic periapical lesions was statistically significant: HCMV ($P = .048$), EBV ($P = .002$), γ -IFN ($P = .001$), IL-1 ($P = .012$), IL-6 ($P = .026$), IL-10 ($P = .026$), IL-12 ($P = .012$), and TNF ($P < .001$; Mann-Whitney *U* test). There was a significant direct correlation between EBV, HCMV, and TNF, γ -IFN, IL-1, IL-6, and IL-12 expression in symptomatic periapical lesions (Spearman). No viruses were detected in the asymptomatic group (Table 3).

Discussion

The present study provides the evidence that herpesviruses participate in the pathogenesis of symptomatic periapical pathosis. The detection of EBV in 8 of 9 (88.9%) and HCMV in 5 of 9 (55%) symptomatic periapical lesions confirmed previous reports that associate EBV and HCMV with marginal and apical periodontitis (1–5, 7). There was a significant direct correlation between EBV,

TABLE 2. Quantification of HCMV, EBV, and Cytokine Expressions by Real-time RT-PCR in Symptomatic Periapical Lesions

Subject no.	Age (y)	Tooth #	HCMV	EBV	TNF- α	γ -IFN	IL-1	IL-6	IL-10	IL-12
1	38	9	2000000	ND	2000000	3000000	ND	3500000	2500000	2000000
2	28	30	ND	4500000	4000000	4000000	ND	3500000	7400000	3000000
3	25	30	800000	300000	5000000	2800000	3000000	3200000	14000000	3000000
4	45	30	ND	3200000	3200000	4500000	40003000	3200000	4000000	3400000
5	25	29	300000	3000000	3400000	2000000	3000000	3400000	ND	4400000
6	60	30	ND	3000000	7500000	11000000	3500000	10000000	4500000	100000
7	25	4	ND	2800000	8000000	7000000	3800000	400000	ND	200000
8	24	5	1200000	200000	3000000	3000000	3200000	8000000	4000000	3000000
9	36	7	700000	300000	2900000	2500000	10000000	10000000	5000000	2000000

ND, not detectable.

HCMV, and TNF, γ -IFN, IL-1, IL-6, and IL-12 expression in symptomatic periapical lesions (Spearman). This finding is partly in accordance with the result of Hernadi et al (8), who demonstrated that EBV DNA were found in apical periodontitis lesions at significantly ($P < .0001$) higher frequencies (72%) than HCMV (10%). Li et al (9) also found both EBV DNA and RNA in irreversible pulpitis, in primary apical periodontitis, and in previously treated apical periodontitis, with significantly higher frequencies when compared with healthy controls. These findings are partly in accordance with our results in detection of EBV; however, our data are not consistent with findings of the other studies that found HCMV occurrence between 0% and 15.9% in apical periodontitis (9–11). It is noteworthy to mention that Ferreira et al reported 61% of the abscess aspirates yielded positive results for viral presence. Human herpesvirus 8 was for the first time detected in high frequency (12). Most previous reports used nonquantitative PCRs for viral detection. Assuming that the amount of virus and cytokine production is an important parameter when evaluating a role in pathogenesis, quantitative real-time PCRs were performed in the present study.

The difference in occurrence of HCMV, EBV, and cytokines between symptomatic and asymptomatic periapical lesions was statistically significant. No viruses were detected in the asymptomatic group, which is in agreement with previous studies reported by Sabeti et al (1–4). It is not known whether the PCR-negative periapical sites harbored the study viruses in a latent stage.

This study neither excludes nor includes the significant role of bacterial flora or infection in the cytokine expression as shown in Tables 2 and 3. However, this study clearly demonstrates the significance of active HCMV and EBV infection (high RNA expression) in key cytokine production. In this context, the comparative analysis in the result section exhibited a parallel marked increase in the cytokines and HCMV and EBV expression in symptomatic periapical lesion when compared with asymptomatic.

Our preliminary studies (unpublished data) are in agreement with other current studies that demonstrate there were various but not specific bacterial types, which indiscriminately were actively present in normal, asymptomatic as well as symptomatic periapical lesions. On this basis, bacterial infection might serve as a cofactor in the

lymphoid transvascular migration and cytokine expression involved in the pathogenesis of periapical lesions.

IL-12 promotes T-helper cell (Th)1 population and suppresses Th2 cell type. Therefore, continuous production of IL-12 might play a role in chronic inflammatory process. In parallel, HCMV and EBV have been shown to exert an antiapoptotic activity. The inhibition of apoptosis might further result in continuous inflammation and cytokine production and establishment of chronic inflammatory stage.

The presence of viral RNA and DNA represents an active inflammatory process. The viral DNA might imply that HCMV or EBV is harbored in cells, or that virus particles are present. The presence of viral RNA implies that active transcription occurs. HCMV and EBV have apparent roles in adhesion, ie, intercellular adhesion molecule expression, nuclear factor kappa B activation, and various chemokine and cytokine production such as IL-1, IL-2, γ -IFN, TNF- α , IL-6, IL-10, IL-12, and prostaglandin E₂ (PGE₂). Herpesviruses can trigger an array of host responses that include dysregulation of macrophages and lymphocytes and serve to down-regulate the antiviral host immune response (13). Host impairment includes silencing of natural killer cells, inhibition of apoptosis, and destruction of components of MHC class I pathway within macrophages, markedly impairing their principal role in antigen presentation (14). In addition, HCMV encodes a unique homolog of IL-10, a Th type 2 cytokine that antagonizes Th1 responses, and its immunosuppressive properties might help HCMV circumvent detection and destruction by the host defense system (15). HCMV also has the ability to inhibit the expression of macrophage surface receptors for lipopolysaccharide, which impairs responsiveness to gram-negative bacterial infections (16). The inhibition of apoptosis might further result in continuous inflammation, cytokine production, and establishment of chronic inflammatory stage.

HCMV reactivation has potential to transactivate EBV, which might constitute a mechanism of increased pathogenicity (17). There was a significant direct correlation between EBV, HCMV, and TNF, γ -IFN, IL-1, and IL-12 in symptomatic periapical lesions.

Herpesvirus infections affect cytokine networks (18). Cytokines and chemokines play important roles in the first line of defense against human herpesvirus infections and contribute significantly to regulation of acquired immune responses. On the other hand,

TABLE 3. Quantification of HCMV, EBV, and Cytokine Expression by Real-time RT-PCR in Asymptomatic Periapical Lesions

Subject no.	Age (y)	Tooth #	HCMV	EBV	TNF- α	γ -IFN	IL-1	IL-6	IL-10	IL-12
1	35	3	ND	ND	ND	ND	ND	1000000	7000	2000000
2	58	13	ND	ND	300000	ND	ND	1000000	6200	2000000
3	30	25	ND	ND	300000	ND	ND	ND	ND	ND
4	50	5	ND	ND	300000	ND	ND	ND	ND	ND
5	55	5	ND	ND	300000	ND	ND	ND	ND	ND
6	35	4	ND	ND	ND	ND	ND	4000000	ND	100000

ND, not detectable.

herpesviruses are able to interfere with cytokine production or divert potent antiviral cytokine responses by a diverse array of strategies, which allow the viruses to survive throughout the lifetime of the host (19, 20). HCMV infection stimulates a Th1-dominance with a proinflammatory cytokine production of IL-1 β , IL-6, IL-12, TNF- α , IFN- α/β , and IFN- γ (18) as well as PGE₂ (21). EBV infection stimulates the production of IL-1 β , IL-1 receptor antagonist, IL-6, IL-8, IL-18, TNF- α , IFN- α/β , IFN- γ , monokine induced by IFN- γ , IFN- γ -inducible protein 10, and granulocyte-macrophage colony-stimulating factor (18). INF- γ , TNF- α , and IL-6 exert particularly high antiviral activity.

Proinflammatory activities basically serve a positive biological goal by aiming to overcome infection or tissue invasion by infectious agents. They can also give rise to detrimental effects with an overwhelming challenge or with a chronic pathophysiological stimulus. In an effort to counteract ongoing inflammation, the proinflammatory response triggers the release of anti-inflammatory mediators such as transforming growth factor- β and IL-10 (22). Herpesviruses display great inventiveness when it comes to diverting potent antiviral cytokine and chemokine responses to their benefit (10). PGE₂, which is a key mediator of the periapical inflammatory response (23), increases rapidly in response to exposure of cells to HCMV, bacterial lipopolysaccharide, and the cytokines IL-1 β and TNF- α (24). PGE₂ might, under certain circumstances, support HCMV replication (25). Undoubtedly, a periapical HCMV infection can induce a multiplicity of interconnected immunomodulatory reactions. Various stages of the infection might display different levels of specific inflammatory cells and mediators, underscoring the complexity of HCMV-host interactions in periapical disease.

In conclusion, we propose that unfavorable changes in environmental exposure or alterations in gene expression of the immune system might periodically suppress the periapical host defense. This then might lead to reactivation of resident herpesviruses and increases in proinflammatory mediators, followed by overgrowth of pathogenic bacteria. A further understanding of the role of herpesviruses in apical infection might be crucial for elucidating the pathophysiology of the disease and for identifying novel and more efficacious targets for disease prevention and long-term cure.

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The authors deny any conflicts of interest related to this study.

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