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Herpesviruses in asymptomatic apical periodontitis lesions: an immunohistochemical approach

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Introduction: Human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) have been recently detected in samples from apical periodontitis lesions by means of molecular biology techniques and a role in the pathogenesis of this disease has been suggested. The present study was designed to survey asymptomatic primary apical periodontitis lesions for the presence of HCMV- and/or EBV-infected cells by means of immunohistochemistry.
Methods: Apical periodontitis lesions were obtained from 35 patients [26 human immunodeficiency virus (HIV) -seronegative patients and nine HIV-seropositive patients] after tooth extraction and subjected to immunohistochemical analysis using monoclonal antibodies specific for HCMV and EBV.
Results: Fifteen of the 35 apical periodontitis lesions were positive for the target herpesviruses. Overall, EBV was found in 31% of the samples and HCMV in 23%, with 14% of the lesions showing EBV and HCMV dual infection. No association was found between HCMV or EBV with any particular histopathological type of apical periodontitis ($P > 0.05$). HCMV was significantly more frequent in apical periodontitis lesions from HIV-positive patients (67%) than in lesions from HIV-negative patients (8%) ($P = 0.001$). EBV was detected in 44% of lesions from HIV-positive patients and in 27% of lesions from HIV-negative patients, but this difference was not significant ($P = 0.91$).
Conclusion: Our results showed that cells infected by HCMV and EBV can be found in apical periodontitis lesions, with a higher prevalence in HIV-positive patients. The specific role that these viruses play in the pathogenesis of apical periodontitis remains to be described.

Key words: apical periodontitis; endodontic infections; Epstein–Barr virus; human cytomegalovirus

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Bacteria have been well established as the main causative agents of apical periodontitis (24). Knowledge of endodontic infections has been significantly refined by the advent of sophisticated culture-dependent and culture-independent methods of microbial identification. The composition of the endodontic microbiota has been unraveled and the current list of candidate endodontic pathogens now includes several bacteria, including *Treponema* spp., *Dialister* spp., *Tannerella forsythia, Porphyromonas* spp., *Prevotella* spp. *Fusobacterium nucleatum, Synergistes* spp., *Peptostreptococcus* spp., *Eubacterium* spp. and *Actinomyces* spp. (15, 23, 25, 30).

Evidence has accumulated over the last decades that suggest the involvement of viruses from the herpesviridae family, especially human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV), with the

pathogenesis of diverse forms of periodontal diseases (1, 8, 9, 17, 26). More recently, molecular biology studies have detected herpesviruses in samples taken from apical periodontitis lesions and a pathogenic role has been suggested (19–22, 28). It has been hypothesized that HCMV and EBV may be implicated in the pathogenesis of apical periodontitis as a direct result of virus infection and replication or as a result of virally induced impairment of local host defenses, which might give rise to overgrowth of pathogenic bacteria in the very apical part of the root canal (28). HCMV and EBV transcripts have been found at high frequencies in the presence of symptoms (20, 22), in lesions exhibiting elevated numbers of anaerobic bacteria (21), and in cases of large periradicular bone destruction (21, 22).

Herpesviruses consist of a single double-stranded DNA molecule enclosed in a viral envelope. Eight human herpesviruses are currently identified: herpes simplex viruses 1 and 2, varicella-zoster virus, EBV, HCMV, human herpesvirus 6, human herpesvirus 7 and human herpesvirus 8 (Kaposi's sarcoma virus) (29). The initial herpesvirus infection is followed by a latent phase in host cells, which may ensure the survival of the viral genome throughout the lifetime of the infected individuals but which has the potential for reactivation (7). Several herpesviruses can infect and influence the function of cells involved with regulation of the immune response (26). HCMV infects monocytes/macrophages, T lymphocytes, polymorphonuclear leukocytes, endothelial cells and fibroblasts and establishes latent infection mainly in bone marrow-derived myeloid progenitor cells. HCMV infection has cytopathological effects that involve nuclear and perinuclear cytoplasmic inclusions, with enlargement of the host cells (cytomegaly), a property that gives the virus its name (3). EBV infects relatively long-lived B lymphocytes during primary infection and during latency, and can also infect the oropharyngeal epithelium. Herpesvirus reactivation may occur spontaneously or as a result of concurrent infection, fever, drugs, tissue trauma, emotional stress, and other factors impairing the host immune defense (27). After activation, herpesviruses can infect several cell types, many of which are commonly found in inflammatory apical periodontitis lesions.

Molecular biology methods have been used to diagnose active herpesvirus infections in apical periodontitis lesions (19–22), but they do not provide information on the spatial distribution of HCMV- and EBV-infected host cells in tissues. Therefore, techniques that associate molecular markers with microscopic analysis, such as immunohistochemistry, would seem to be useful in the study of the anatomy of the infectious process. Based on these premises, this study was undertaken to investigate the occurrence of host cells infected by HCMV and EBV in asymptomatic apical periodontitis lesions using an immunohistochemical approach.

Materials and methods

Collection of apical periodontitis specimens

Biopsy samples of asymptomatic apical periodontitis lesions were obtained from 35 patients [26 human immunodeficiency virus (HIV) -seronegative and nine HIV-seropositive patients] who were seeking surgical treatment at the Surgical Clinic of the Faculty of Dentistry, Estácio de Sá University. Each patient contributed an extracted tooth associated with an apical periodontitis lesion. All teeth were free of spontaneous symptoms and negative to percussion and palpation at the time of extraction. Teeth were extracted for prosthetic reasons and had extensive carious lesions and radiolucent apical lesions, which remained attached to the root tips after extraction. The size of the apical periodontitis lesions ranged from 3×3 mm to 12×13 mm. None of the teeth had periodontal pockets deeper than 3 mm. Before extraction, patients rinsed with 0.12% chlorhexidine mouthwash for 30 s. Afterwards, the tooth, gingiva and mucosa of the sampled area were scrubbed with 0.12% chlorhexidine. Immediately after extraction, the teeth were washed with sterile saline solution and the lesions were detached using a sterile #15 scalpel blade. Lesions were fixed by immersion in 10% formalin solution and were then paraffin embedded. Specimens were evaluated by histological staining for histopathological classification of the lesion and immunohistochemistry for detection of virus-infected host cells. Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Histopathological classification

Serial 6-μm histological sections were obtained from the lesions. Some sections from each lesion were stained with hematoxylin & eosin and with Gomori's trichrome. Sections were analyzed on a light microscope (Olympus-BH2-RFCA, Tokyo, Japan) equipped with a digital camera (Sony-CCDDXC151-A, Tokyo, Japan) coupled to a computerized analysis system (IMAGE-PRO version 1.2, Media Cybernetics, Tokyo, Japan). Based on histopathological analysis, apical periodontitis lesions ($n = 35$) were categorized as granuloma, epithelialized granuloma or cyst.

Immunohistochemical analysis

Immunohistochemical procedures were performed using a standardized protocol based on the streptavidin–biotin complex technique (32). After dewaxing and rehydration, sections were immersed in 0.01 mM citrate buffer (pH 6.0) for 40 min at 95°C for antigen retrieval. Sections were incubated for 20 min in 3% hydrogen peroxide in methanol at room temperature to block endogenous peroxidase activity, and were then washed in distilled water and phosphate-buffered saline (pH 7.6). Afterwards, the sections were incubated in normal goat serum for 10 min at room temperature and then incubated overnight in a humid chamber with the following primary monoclonal mouse antibodies: anti-HCMV M0854, which is a mixture of two monoclonal antibodies – CCH2 and DDG9 (DakoCytomation, Glostrup, Denmark) and anti-EBV M0897, which is a mixture of four monoclonal antibodies – CS.1, CS.2, CS.3 and CS.4 (DakoCytomation) (Table 1). Subsequently, sections were incubated with a biotinylated secondary antibody for 15 min followed by incubation with streptavidin–biotin–peroxidase complex, also for 15 min (LSAB kit, DakoCytomation). The peroxidase reaction was carried out using a substrate–chromogen system solution containing diaminobenzidine and hydrogen peroxide (Liquid DAB + Substrate Chromogen System, Dako-K3468, DakoCytomation). Finally, sections were counterstained with Harris's hematoxylin

Table 1. Primary antibodies used for immunohistochemical staining of asymptomatic apical periodontitis lesions

Antibody	Source/Type	Reactivity	Dilution
Human cytomegalovirus M0854	Mouse monoclonal/DDG9 Mouse monoclonal/CCH2	76,000 MW ptn DNA-binding ptn (p52)	1 : 50
Epstein–Barr virus LMP M0897	Mouse monoclonal/CS1-4	60,000 MW LMP-1[1]	1 : 250

[1]Latent membrane protein-1.

and mounted with synthetic resin. All washing steps were carried out in phosphate-buffered saline (pH 7.6) at room temperature. Positive controls for both viruses were performed using histological sections of human lymphoma from patients with a history of infection by the target herpesviruses.

All immunostaining procedures were performed in triplicate. Intracellular deposits (cytoplasmic or nuclear) were defined as homogeneous when all the nucleus or cytoplasm was stained and heterogeneous when there were differences in the staining intensity or there were visible patches.

Statistical analysis

The prevalence of HCMV and EBV in asymptomatic apical periodontitis lesions was recorded as a percentage of the cases examined. The following comparisons were performed: overall prevalence of the two herpesviruses; prevalence of these two herpesviruses taking into account the different histopathological classification of the lesions; and prevalence of HCMV and EBV in lesions from HIV-positive and HIV-negative patients. For statistical analysis, either the chi-squared test with Yates's correction or the Fisher's exact test was used. Significance levels were established at 5% ($P < 0.05$).

Results

Fifteen out of 35 apical periodontitis lesions demonstrated positive staining for the target herpesviruses. Overall, EBV was found in 11 samples (31%) and HCMV was found in eight samples (23%), regardless of the histological classification of the lesion. There was no significant difference when comparing the prevalence of these two viruses in asymptomatic apical periodontitis lesions ($P = 0.29$). Histopathological analysis of the specimens ($n = 35$) revealed 22 granulomas, seven epithelialized granulomas and six cysts (Table 2; Fig. 1). No association was found between HCMV or EBV with any particular histological type of apical periodontitis lesion ($P > 0.05$).

In most EBV-positive specimens, infected cells were sparsely distributed and most of them showed weak heterogeneous or homogeneous staining of the cytoplasm (Fig. 2). However, two specimens exhibited cells with intense, homogeneous cytoplasmic staining. EBV-infected cells showed abnormal morphology. They were larger than normal, amebiform or oval and some of them had an altered nuclear shape; in these cells, the nucleus was irregular with evincing large indentations and exhibiting egg-shaped or folded forms (Fig. 2).

HCMV-infected cells showed both nuclear and cytoplasmic staining (Fig. 3). These cells presented a weak to intense homogeneous staining. Many infected cells exhibited larger sizes when compared to non-infected cells. HCMV-infected cells were distributed throughout the connective tissue and sometimes on vascular walls, including arterioles and venules (Fig. 3). HCMV-positive cells dispersed through the connective tissue resembled lymphocytes, fibroblasts or macrophages, while the cells at vascular walls were probably those that structurally composed the vascular tunica, including endothelial cells, smooth muscle cells and vascular fibroblasts. Signs of nuclear shape alterations and disorganization were observed in these cells (Fig. 3).

HCMV was significantly more frequent in lesions from HIV-positive patients (six out of nine cases, 67%) than in lesions from HIV-negative patients (two out of 26 cases, 8%) ($P = 0.001$; Table 3). EBV was detected in four out of nine (44%) lesions from HIV-positive patients and in seven out of 26 (27%) lesions from HIV-negative patients (Table 3). This difference was not significant ($P = 0.91$). Five of the 35 apical periodontitis lesions showed a dual infection with both EBV and HCMV (14%). Three of these samples were from HIV-positive patients (33% of the HIV-positive samples; Table 4).

Discussion

Apical periodontitis has long been established as a group of inflammatory disorders evoked by bacteria (24). Recently, a series of studies have suggested viral participation, more specifically herpesviruses, in the development and evolution of periradicular inflammatory diseases (19–22). Those studies used polymerase chain reaction technique to demonstrate the presence of transcripts of EBV and HCMV in both symptomatic and asymptomatic apical periodontitis lesions, with higher prevalence values in the former (20, 22). Although such an association does not necessarily translate into causation, such results open another route of thought that may at least provide complementary explanations about the etiology of different types of apical periodontitis.

The search for EBV and HCMV in apical periodontitis lesions had thus far been limited to molecular genetic analysis. Although polymerase chain reaction techniques are a powerful tool for the direct detection of pathogens in biological samples, they fail to show the spatial distribution of the infectious agents and their relationship with tissue structural components and cells. In the present study, we succeed in identifying herpesvirus-infected cells using immunohistochemistry in some asymptomatic chronic apical periodontitis lesions, in addition to showing their distribution through the inflamed periradicular tissues.

Our overall data revealed that EBV-infected cells occurred in 31%, and HCMV-infected cells in 23%, of the cases of asymptomatic apical periodontitis lesions. Co-infection was found in 14% of the cases. Cytoplasmic staining was observed for HCMV-infected cells, which is typical of the late stages of infection (16, 18). Even though molecular identification studies revealed that herpesvirus are detected significantly more often in symptomatic apical periodontitis lesions, our findings confirmed the occurrence of EBV- and HCMV-infected cells in asymptomatic lesions. It should be pointed out that most symptomatic infections had their origin in asymptomatic lesions. Therefore, it is fair to assume that should herpesviruses be involved with symptomatic lesions, at some point the infection will develop in asymptomatic lesions preceding the development of symptoms. However, in almost all positive specimens, infected cells were usually sparse and represented by few positively stained cells, which were usually in close physical proximity and were concentrated in areas that were no more than 1 mm in diameter. Whether these mild infections could give rise to further symptomatic conditions and which

Table 2. Histological classification of the examined apical periodontitis lesions

Lesion	HIV-negative patients	HIV-positive patients	Total
Granuloma	16	6	22
Epithelialized granuloma	6	1	7
Cyst	4	2	6
Total	26	9	35

HIV, human immunodeficiency virus.

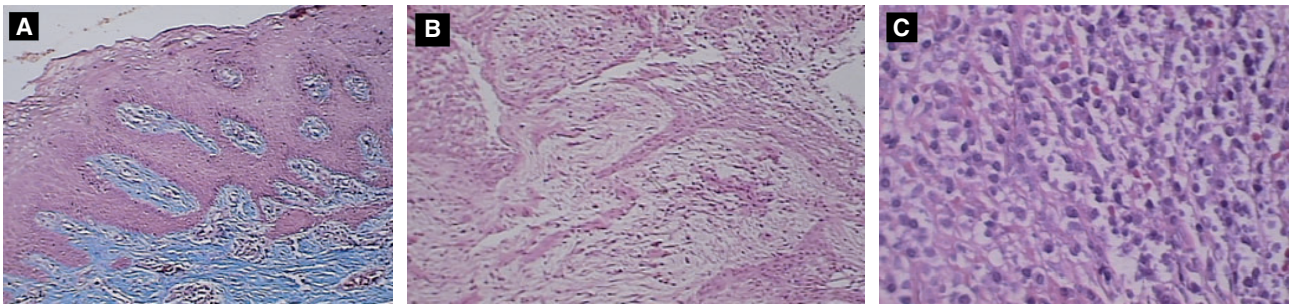

Fig. 1. Histopathological classification of the examined asymptomatic apical periodontitis lesions. (A) Cyst, original magnification × 100; (B) epithelialized granuloma, original magnification × 100; (C) granuloma, original magnification × 400.

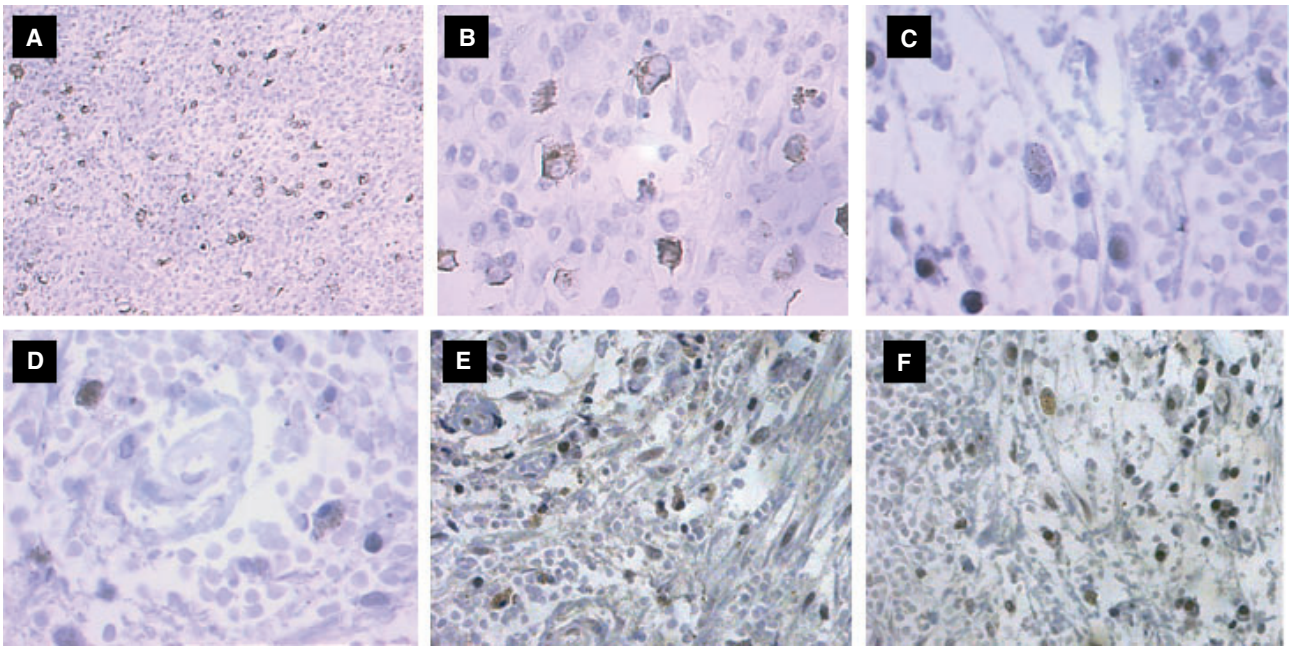

Fig. 2. Specimens showing EBV-positive staining (anti-EBV staining, counterstained with Harris's hematoxylin). (A) Positive control: histological section of lymphoma (original magnification × 100); (B) detail of A (× 400); (C) to (F), granuloma specimens showing EBV-positive staining. Note the sparse pattern of infected-cell distribution. (C), EBV-positive cell with folded nucleus (× 630); (D) positive cells with modifications in the shape of the nucleus (× 630); (E, F) several EBV-infected cells distributed through the tissue showing alterations in both cellular morphology and nuclear form, with intense cytoplasmic staining (× 400).

factors could influence such a transition remain to be elucidated.

It is entirely possible that at a given time, herpesvirus-infected cells are attracted to the apical periodontitis lesion. If for any reason bacterial aggression coming from the root canal increases, a large number of immunological cells can be attracted to the periradicular tissues to counteract bacterial invasion and aggression. This increases the possibility of virus-infected cells arriving and accumulating at the apical periodontitis lesion and helps to explain the higher incidence of these viruses in symptomatic lesions as reported by molecular biology studies (20, 22).

An interesting finding from this study was that EBV- and HCMV-infected cells were more frequent in apical periodontitis lesions from HIV-seropositive subjects than in HIV-seronegative subjects, but only HCMV data reached statistical significance ($P = 0.001$). This confirms that the incidence of herpesvirus infection is closely related to immunosuppressive state (13). HCMV has been detected in 81% of HIV periodontitis lesions (1). Herpesvirus-associated diseases are common in the mouths of HIV patients and are often indicators of severe immunosuppression (6). It has been demonstrated that herpesvirus reactivation in patients infected with HIV can result in severe herpesvirus infections (2, 5, 13). Indeed, immunosuppression induced by HIV infection is known to predispose to herpesvirus reactivation (4). In HIV-positive patients, co-infection by EBV/HCMV also increases the potential for herpesvirus reactivation (31). Moreover, it has been claimed that herpesviruses can interact with HIV at the cellular and molecular level to accelerate the rate of periodontal tissue destruction (1, 5, 13). In fact, herpesvirus infections in HIV-seropositive patients may accentuate local immune suppression, impair protective immunity, induce proinflammatory cytokine production, alter the structural integrity of the periodontal tissues, and lead to overgrowth of associated bacteria (1). All these effects might also be of significance for the pathogenesis of apical periodontitis.

The hallmark of herpesvirus infections is immune impairment and permanence, remaining for long periods in a latent state

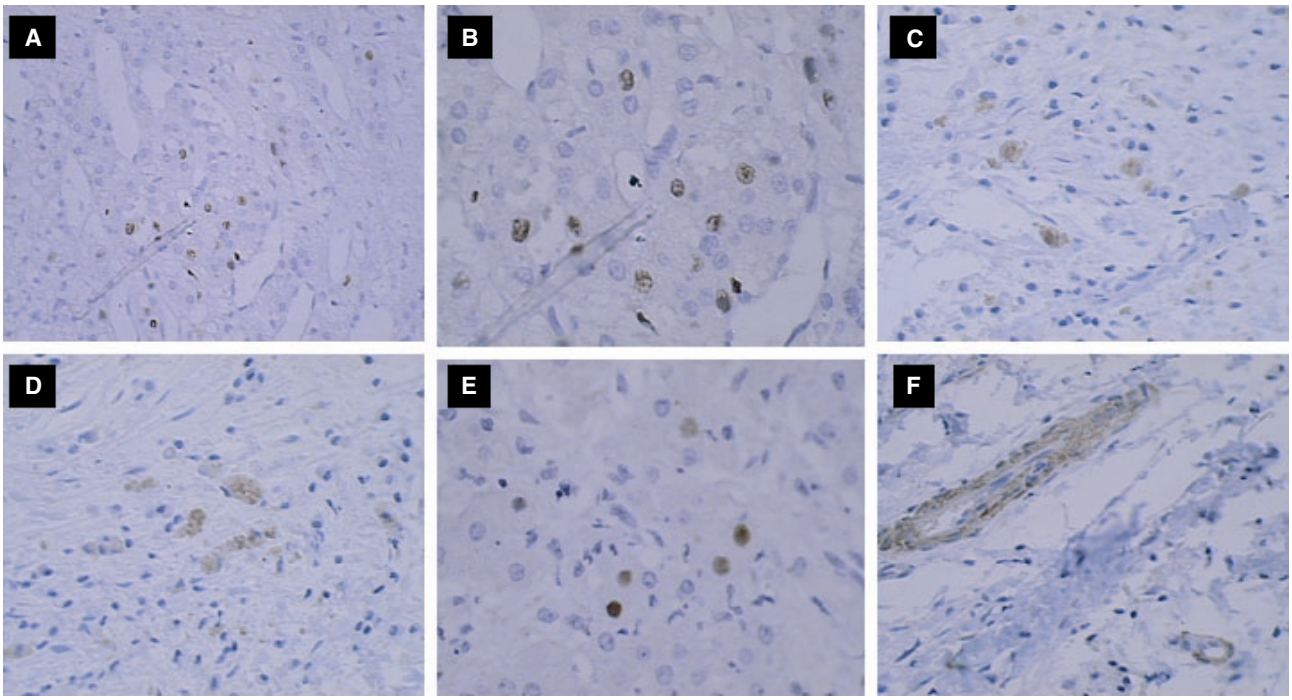

Fig. 3. Specimens showing HCMV-positive staining (anti-HCMV staining, counterstained with Harris's hematoxylin). (A) Positive control: histological section of lymphoma (original magnification × 200); (B) detail of A (original magnification × 400); (C, D) HCMV-positive cells showing alterations in cellular morphologies and intense nuclear and cytoplasmic staining (original magnification × 400); (E) HCMV-positive cells showing intense nuclear staining (original magnification × 400); (F) cyst showing perivascular HCMV-positive cells with cytoplasmic staining (original magnification × 400).

Table 3. Occurrence of cytomegalovirus-positive and Epstein–Barr virus-positive cells in 35 apical periodontitis lesions and correlation with human immunodeficiency virus infection

Lesion	EBV-positive (Total)	EBV-positive (HIV patients)	HCMV-positive (Total)	HCMV-positive (HIV patients)
Granuloma	8/22	3/6	5/22	4/6
Epithelialized granuloma	2/7	0/1	2/7	1/1
Cyst	1/6	1/2	1/6	1/2
Total	11/35	4/9	8/35	6/9

EBV, Epstein–Barr virus; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus.

Table 4. Occurrence of human cytomegalovirus and Epstein–Barr virus co-infection in 35 apical periodontitis lesions and correlation with human immunodeficiency virus infection

Lesion	Co-infection EBV/ HCMV (Total)	Co-infection EBV/ HCMV (HIV patients)
Granuloma	3/22	2/6
Epithelialized granuloma	1/7	0/1
Cyst	1/6	1/2
Total	5/35	3/9

EBV, Epstein–Barr virus; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus.

in host tissues (26). After activation, various herpesviruses can infect monocytes and macrophages, T and B lymphocytes, epithelial cells, endothelial cells, fibroblasts and other mammalian cells. Alternation between prolonged periods of virus latency interrupted by periods of activation may be hypothesized as being partly responsible for the intermittent symptoms of apical periodontitis. Furthermore, the recurrence or persistence of some apical periodontitis lesions despite treatment might be partly caused by the deficient elimination of bacteria invading the periradicular tissues as a result of virus-induced reduction in the ability of the immune system to handle bacterial infection.

An individual who has had previous contact with EBV and/or HCMV may develop a latent infection by these viruses. Bacterial challenge emanating from the canal may cause an influx of virus-infected cells into the periradicular tissues. Reactivation of HCMV and/or EBV by tissue injury induced by bacteria may evoke the impairment of the host immune response in the periradicular microenvironment, changing the potential of local defense cells to mount an adequate response against infectious agents. Histopathological features of apical periodontitis lesions include active participation of cells, many of which are potentially susceptible to infection by EBV and HCMV (10–12). Moreover, herpesvirus-infected inflammatory cells are stimulated to release tissue-destroying cytokines (14, 33), which may exacerbate inflammation at the periradicular tissues and lead to symptoms.

It is salient to point out that our findings do not represent a confirmation of the hypothesis of the role played by EBV and HCMV in apical periodontitis causation. This is just a descriptive study that found EBV- and HCMV-infected cells in asymptomatic primary apical periodontitis. At this time, and based on these and other findings, it is difficult to ascribe a specific role for herpesviruses in the pathogenesis of apical periodontitis. It is apparent from our findings that the presence of herpesvirus in the periradicular tissues of

asymptomatic lesions is a result of the influx of infected inflammatory cells into the area. Whether these viruses are activated, and thereby are directly or indirectly related to the pathogenesis of apical periodontitis lesions, remains hypothetical. Therefore, herpesvirus contribution to the development of apical periodontitis is still an open question and additional studies are needed to elucidate this issue.

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