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Comparison of the occurrence of cytomegalovirus and epstein-barr virus infection in periradicular lesions of HIV-positive and HIV-negative patients: an immunohistochemical study

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Introduction

The main pathological changes that affect the periradicular tissues are of an inflammatory nature and of infectious etiology. From the moment the pulp tissue loses its vitality and, consequently, its defense and regeneration potential, microorganisms invade and infect the root canal system, establishing an endodontic infection. The consequence of this process is the establishment of a microbiota dominated by strict anaerobic bacteria that in most cases generate an inflammatory periradicular lesion.[1] Once the aggressive stimulus persists, the host defense responses, specific and unspecific, can generate damage to the periradicular tissues, where microorganisms are the main triggering agents of the entire pathogenic process.[2]

The association of bacteria with endodontic infections was confirmed by a classic study that demonstrated that, after exposure of the pulp chamber to the oral cavity and consequent pulp necrosis, the periradicular tissues were compromised only in conventional animals and not in germ-free animals.[3] From then on, bacteria and fungi became the main pathogens involved in pulp and periradicular pathologies, especially: *Fusobacterium nucleatum, Porphyromonas endodontallis, Tannerella forsythia, Propionibacterium propionicum, Campylobacter rectus, Peptostreptococcus micros, Prevotella nigrescens, Prevotella intermedia, Enterococcocus faecalis*, species of *Treponema* and *Candida albicans*.[4-6] According to Siqueira,[7] variations in host resistance and micro-environmental changes in the root canal system, such as nutrient demand, pH, oxygen tension, microbial interactions, presence of virulent strains in sufficient quantity, in addition to resistance of the host, can trigger the appearance of periradicular lesions or even an acute inflammatory response. The presence of pathogenic microorganisms is crucial for the development of periradicular disease, but their mere presence does not seem to provide sufficient data to explain important clinical-pathological characteristics of the disease. It is still unknown why the periradicular tissues, in some cases, remain intact, despite pulp necrosis or even the event that predisposes to an acute condition in chronic lesions that remain asymptomatic for years.

Apparently, herpesviruses can significantly contribute to the etiopathogenesis of several types of periodontal diseases, since the DNA of these viruses has been frequently detected in localized, generalized, chronic, aggressive periodontitis[8-10] in gingivitis and acute necrotizing ulcerative gingivitis in adults and children.[11] In recent years, studies have also investigated the presence of human herpesviruses in patients with periradicular lesions.[12-14] According to recent findings regarding the involvement of herpesviruses in periodontitis and periradicular pathologies together with specific bacteria, it has been suggested that Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) are potential pathological agents related to tissue destruction in both diseases.

Literature review

According to Slots,[15] viruses occupy a unique position in biology. They are obligate intracellular agents and therefore are metabolically and pathologically inert when outside the host cell. Although viruses have some properties such as genome and ability to replicate, they are definitely inanimate infectious agents and should not be considered as microorganisms.

The eight human viruses of the *Herpesviridae* family (Herpes simplex type 1–HSV-1, Herpes simplex type 2–HSV-2, Varicella Zoster–VZV, Epstein-Barr virus–EBV, Cytomegalovirus–HCMV, Herpes human virus 6–HHV-6, Human Herpes Virus 7–HHV-7, Human Herpes Virus 8–HHV-8) may be present and infect the majority of the population. Despite being known for decades and being well characterized, little is known about the pathogenic potential of members of this family.[16,17]

All viruses are basically composed of: nucleic acid (DNA or RNA), and a protective protein membrane known as a capsid. The set formed by the genome and the capsid is called the nucleus-capsid. Some viruses have an additional coat in the form of an envelope that consists of a lipoprotein bilayer derived from the cell membrane of the host cell.[15]

Members of the *Herpesviridae* family, especially HCMV and EBV, are structured from 4 virion structures (viral particle found outside the host cell). Herpesviruses have a core containing an isolated double strand of DNA surrounded by an isopentahedron capsid, which has

162 capsomers. An amorphous proteinaceous tegument is still part of the viral structure and surrounding the capsid and tegument, a lipid bilayer derived from the host cell membrane. The viral envelope contains glycoproteins that act as bonds in cellular coupling and are targets of important reactions of the host immune system.[15]

Human herpesviruses are classified into three different groups (α-HSV-1, HSV-2 and VZV; β-HCMV, HHV-6 and HHV-7; γ-EBV and HHV-8) according to their pathogenicity, behavior in culture and tendency to react in a specific way to a tissue or stimulus (tissue tropism).

Herpesvirus transmission can occur vertically, that is, from mother to child during childbirth or prenatal care, or horizontally through person-to-person contact. The viruses of the *Herpesviridae* family can be found in urine, vaginal secretions, semen, breast milk, feces and blood.[18,19] Saliva in immunocompromised patients can present a range of herpesviruses and can often serve as a vehicle for viral transmission.[18] Viruses are related not only to numerous infectious diseases, but also to several types of cancer in humans.[20-22]

Primary herpesvirus infection in an immunocompetent host usually causes mild symptoms. After primary infection, herpesviruses remain latent in the host, having the potential to generate disease if the virus reactivates, especially in an immunocompromised host, such as a patient also carrying the human immunodeficiency virus (HIV). In contrast, reactivation of herpes viruses in a non-immunocompromised patient is rare.[23]

Diseases related to EBV and HCMV herpes viruses

There are approximately 120 different species of herpes virus. However, it is known so far that only 8 types can infect humans.[15]

Epstein-Barr virus (EBV) infection usually occurs through contaminated saliva.[24] EBV is the etiologic agent of mononucleosis, a disease that causes persistent fever, cervical lymphadenopathy, in addition to oral lesions and petechiae on the hard palate in about 25% of patients.[24] EBV is also related to the pathogenesis of Hodgkin's disease or Hodgkin's lymphoma, a malignant lymphoproliferative disorder also defined as a neoplasm of B lymphocytes. Serological, epidemiological, and molecular studies suggest the presence of elevated levels of EBV in patients with Hodgkin's lymphoma.[25-28] According to Kaya et al.,[25] patients with a history of mononucleosis demonstrated a greater predisposition to develop Hodgkin's disease, in addition to having high levels of antibodies to EBV. According to Walling,[29] hairy leukoplakia is related to EBV replication. Inhibition of replication with antiviral therapy results in disease resolution in about 1 to 2 weeks. According to the same author, EBV replication is necessary, but other factors that are not yet well defined are involved in the pathogenesis of the disease. Several studies also point to EBV as one of the etiological factors in diseases such as nasopharyngeal carcinoma, a malignancy present in the lining epithelium of the nasopharynx[30,31] and Burkkit lymphoma, a malignancy originating in the B lymphocyte.[32,33]

According to Distéfano et al.,[19] among the main diseases related to Cytomegalovirus (HCMV) infection, congenital impairments are highlighted, resulting from the transmission of the virus through the placental wall, which can cause several neurological sequelae (mental retardation, hearing loss, motor deficiencies, epilepsy and etc). Encephalitis also stands out as one of the diseases resulting from HCMV infection, not only in neonates and transplant patients, but mainly in HIV patients with low CD4 cell counts.[34] Some studies highlight the fact that the reactivation of viral replication and consequent immunosuppression is one of the factors that leads to organ rejection in transplant patients when they are infected with HCMV.[35,36]

Involvement and epidemiology of HCMV and EBV in periodontal diseases

Despite a long history of studies seeking to elucidate the etiopathogenesis of marginal periodontitis, many doubts remain unresolved. The presence of pathogenic microorganisms is crucial for the development of periodontal disease, but the simple presence of bacterial plaque does not seem to provide enough data to explain important clinical-pathological characteristics of the disease.[37] Many uncertainties have prompted efforts to identify additional etiologic factors for periodontal disease. It is still unknown the reason why periodontal tissues remain intact in adverse conditions, or even the event that predisposes to an acute condition in chronic lesions or rapid bone loss in aggressive lesions.[38]

Important advances regarding the recognition of the infectious agents of periodontal disease have occurred in the last decades. However, in recent years, studies have investigated the presence of human herpesviruses in patients with periodontal disease.[39,40] Herpesviruses apparently contribute significantly to the etiopathogenesis of several types of periodontitis, since the DNA sequence of these viruses has been frequently reported in localized, generalized, chronic, aggressive periodontitis and in cases of gingivitis and acute necrotizing ulcerative gingivitis in adults and children.[14]

According to recent findings regarding the involvement of herpesviruses in periodontal disease together with bacteria and the host's immune response, it has been suggested that HCMV and EBV are pathological agents that determine tissue destruction in different forms of periodontal diseases.[15]

Contreras et al.,[11] studied the relationship between herpesviruses and acute necrotizing ulcerative gingivitis (GUNA) in nourished and malnourished Nigerian children aged 3-14 years. The authors examined 62 children of whom 22 were malnourished and had GUNA, 20 were malnourished but free of GUNA, and the remaining 20 were well-nourished and also with healthy oral conditions. The specimens were collected through sterile absorbent paper points that were inserted between the tooth and the affected gingival tissue for 20 seconds for further molecular analysis. The results presented in the study reveal that of the group of children with GUNA, 59% were infected with HCMV, while EBV-1 infected 27%, and HSV and HHV-6 were detected in 23% and 5% of children, respectively. The results also revealed that the investigated herpesviruses were significantly more prevalent in children with GUNA than in those free of the disease.

Contreras et al.,[8] performed another study investigating the presence of HCMV, EBV-1, HSV and HHV-6 in excised biopsies of periodontal pockets. For the study, 20 samples were collected from patients who had periodontal disease and 3 samples from healthy patients. According to the results of the study, in 18 (90%) of the 20 specimens collected that had periodontal disease, the presence of herpesvirus could be identified. HCMV was detected in 13 samples (65%), EBV in 10 (50%), HSV in 7 (35%) and HHV-6 in 2 samples (10%). Co-infection was detected in 12 samples (60%), being the combination HCMV + EBV in 7 specimens (35%). Of the 3 healthy samples, only one (33%) showed infection with herpesvirus, HSV. In the same study, the authors sought to identify cells infected by the aforementioned herpesviruses in 20 specimens. The results revealed that 14 specimens (70%) had infected cells. Monocytes/macrophages

infected by HCMV and HSV were found in 11 (55%) and 01 (5%) of the specimens respectively collected. HCMV was detected infecting T lymphocytes in 4 specimens (20%), same number for HSV. Nine specimens (45%) had EBV-infected B lymphocytes. A single sample showed a monocyte/macrophage co-infected with HCMV + HSV.

Contreras et al.,[41] examined the relationship between the presence of Herpesvirus and periodontal disease in 140 adults with gingivitis or periodontal disease. According to the classification criteria performed by the authors, 30 patients had severe periodontitis, 38 had moderate periodontitis, 31 had mild periodontitis and 41 had gingivitis. The samples were collected through sterile absorbent paper points that were inserted for twenty seconds into the deeper periodontal pockets for further molecular analysis. The results presented by the study revealed that 53% of patients with severe periodontal disease had herpesvirus-infected pockets, while patients with moderate, mild periodontitis and gingivitis had 45%, 42% and 17% of infected periodontal pockets, respectively. Of the total of 140 samples, 53 were found to be infected. HCMV was the most common herpesvirus (32 samples) followed by EBV-1 (18 samples), HSV (11 samples) and EBV-2 (06 samples). In the same study and in agreement with the results, the authors report that there is still a significant relationship between HCMV and EBV-1 with elevated levels of *Porphyromonas gingivalis*, and other putative microorganisms including *Tannerela forsythia, Prevotella intermedia, Prevotella nigrescens* and *Treponema denticola*.

Contreras et al.,[39] carried out a study to identify the presence of HCMV, EBV and other herpesviruses in periodontal pockets and gingival tissue of 25 patients. Of the 25 patients, 14 had periodontitis while the remaining 11 patients had healthy periodontal tissues. According to the results, EBV-1 was identified in 2 samples collected from healthy periodontal pockets while HCMV was identified in only one. Biopsies of healthy gingival tissue revealed the presence of EBV-1 and HCMV in 3 and 2 samples, respectively. Samples collected from periodontal pockets and gingival tissue from patients with periodontal disease revealed that EBV-1 was present in 6 and 11 cases, respectively, while HCMV was identified in 9 pockets and 12 gingival specimens.

Seeking to evaluate the occurrence of herpesvirus in immunocompromised and non-immunocompromised patients with periodontal disease, Contreras et al.,[42] excised gingival tissue from periodontal pockets in 21 seropositive and 14 seronegative patients. The results confirm a high incidence of herpesvirus in periodontal pockets of compromised patients. In HIV patients, HCMV was detected in 81% of samples, HHV-6 was detected in 71% of samples, HHV-7 in 67%, EBV-1 and 2 in 57% each, HSV in 43% and HHV-8 at 24%. In seronegative patients, the results were 50% for HCMV and EBV-1, 43% for HSV, 29% for HHV-7 and 21% for HHV-6. HHV-8 and EBV-2 were not reported in healthy patients. According to the authors, active herpesvirus infection in HIV patients with periodontal disease may result in a decrease in the host's local immune defenses. This damage to the immune system would favor the multiplication of pathogenic microorganisms and consequently magnify the destruction of periodontal tissues.

In a study aimed at evaluating the occurrence of human herpesviruses in patients with periodontal disease, Kamma et al.,[43] collected subgingival plaque samples from 16 patients. Initially, patients received periodontal treatment, including oral hygiene instructions, scaling, root planing, periodontal surgery, and systemic antibiotic prescription. After completion of initial therapy, patients were scheduled for maintenance appointments every 3-6 months. At the end of the maintenance period, samples were collected using endodontic absorbent paper points inserted into predetermined periodontal pockets after a thorough clinical examination. According to the study criteria, 64 samples were collected, 4 per patient (2 from bags with active disease and 2 from bags with controlled disease). Viral results revealed that of the 32 pouch samples with active disease, 19 (59%) were infected with HCMV, 14 (44%) with EBV-1, and 11 (34%) with HSV. On the other hand, of the 32 bags with controlled disease, only 4 (12.5%) were infected by HCMV, 4 (12.5%) by EBV-1, and 3 (9%) by HSV. Co-infection of 2-3 viruses was detected in 14 (44%) active pouches, and in only one (3%) stabilized pouch. According to the conclusion of the study, mono-infection or co-infection with herpesviruses is significantly associated with active periodontitis.

Saygun et al.,[44] sought to determine the occurrence of HCMV, EBV-1 and HSV in patients with chronic periodontal disease and the relationship between these herpesviruses and clinical parameters of the disease. Thirty subgingival samples from patients with chronic periodontitis and 21 randomly selected and collected healthy samples were analyzed by molecular method. The results revealed that HCMV was detected in 44% of patients with chronic periodontitis and in 14% of patients with healthy pockets. EBV-1 was detected in 17% and 14% of patients with periodontal disease and healthy patients, respectively. HSV infected 7% of samples from patients with periodontitis, whereas in healthy patients no samples were found to be infected. The results of this study confirm the frequent presence of HCMV and EBV-1 in patients with chronic periodontal disease, suggesting a strong relationship between these herpesviruses with deep pockets, bleeding and significant loss of bone attachment.

Yapar et al.,[10] seeking further evidence of the role of herpesviruses in the pathogenesis of periodontal disease, carried out a study evaluating the presence of HCMV and EBV in patients with aggressive periodontitis. Seventeen patients with aggressive periodontal disease and 16 healthy patients were selected. According to the diagnostic criteria of the study, patients were considered to have aggressive periodontitis when they were younger than 35 years, and exhibited extensive periodontal destruction, bone loss exceeding 5 mm in 2 or 3 regions in more than 14 teeth (except first molars and incisors). Due to the severity of the disease, all compromised patients underwent surgical treatment and systemic antibiotic therapy. Samples were collected 3 months after surgery using sterile curettes. The results revealed detection of viral DNA in both groups. Of the patients with periodontitis, 65% (11 samples) were infected with HCMV while EBV-1 was detected in 12 samples (71%). Of the healthy patients, only EBV-1 was detected in a single sample (6%). In the same study, it can be concluded that both herpesviruses are closely associated with the pathogenesis of the disease, as co-infection appears to be extremely deleterious to periodontal tissues.

Seeking to elucidate a link between HCMV and EBV with periodontal abscesses, Saygun et al.,[45] performed a study where 18 patients with periodontal abscess were selected. Viral samples were collected from sterile curettes inserted to the bottom of the periodontal pocket and removed in a single movement in order to remove subgingival debris. A sample from the abscess region and another sample from a homologous healthy region was collected from each patient for further molecular analysis. The results of the study reveal that in the abscessed regions, HCMV and EBV were detected in 12 (67%) and 13 (72%) samples, respectively. In samples collected from healthy regions, HCMV was detected in only one (6%) sample while EBV was not detected in any healthy sample. Co-infection was reported in 10 (56%) of 18 samples collected from regions with periodontal abscess. Four months after treatment and antibiotic

therapy, the same abscessed and healthy sites were investigated in an attempt to identify the presence of the respective herpesviruses. However, HCMV and EBV were not detected in any of the samples collected.

Saygun et al.,[46] conducted a study to assess the interrelationship of human herpesviruses with pathogenic bacteria of periodontal disease. After supragingival plaque removal, subgingival samples were collected using sterile curettes from 18 patients with periodontal disease and 16 healthy patients. According to the results presented in the study, HCMV, EBV-1 and HSV-1 were detected in 72-78% of specimens from patients with periodontal disease while the microorganisms *P. gingivalis*, *T. forsythia* and *C. rectus* were reported in 78-83% of samples. *P. intermedia* and *Actinobacillus actinomycetemcomitans* were each demonstrated in 44% of samples from compromised periodontal pockets. In patients with healthy periodontal tissues, EBV-1 was reported in only one sample, as were *P. gingivalis* and *A. actinomycetemcomitans*. The species C. rectus was demonstrated in 3 samples. In the same study, the authors reported a statistically significant occurrence between herpesviruses and bacteria. Strong association was found between HCMV and HSV-1 with *P. gingivalis*, *P. intermedia*, *T. forsythia* and *C. rectus*, and between EBV-1 with *P. gingivalis*, *T. forsythia*, and *C. rectus*. HSV-2 showed no link with any bacterial species.

In a study whose objective was to identify levels of HCMV and EBV infection in periodontal pockets and adjacent gingiva using *Real-Time* PCR, Kubar et al.,[37] selected 20 patients with periodontal disease of which 9 had aggressive periodontitis and the remainder had chronic periodontitis. After removing plaque and supragingival calculus with ultrasonic curettes, samples of subgingival and adjacent inflamed gingiva were collected from each of the 20 patients using sterile manual curettes. Samples were obtained from the deepest periodontal pocket (6-10 mm). Results revealed that more than 10,000 copies of HCMV or EBV were detected in the periodontal pocket or gingival tissue in 7 of 9 patients with aggressive periodontitis and in 3 of 11 patients with chronic periodontitis. Of the 13 patients with a periodontal pocket larger than 6 mm, 10 showed more than 10,000 copies of HCMV or EBV in the subgingival or gingival tissue samples, while the same value was not detected in the patients with a 6 mm pocket. The highest herpesvirus counts were reported in patients 1, 2 and 19, who also had the highest severity of periodontal disease. The results also demonstrated a strong association between HCMV and EBV counts with the most severe periodontal conditions.

According to the various existing data, much information is consistent in revealing the participation of herpesviruses in the etiology and pathogenesis of various periodontal diseases. Herpesviruses can generate periodontal pathologies as a direct result of infection and replication by the virus or indirectly causing damage to the host's defenses. Herpesviruses can exert their pathogenic effects on periodontal tissues through some mechanisms that operate alone or in combination.[9] They can generate cytopathic effects on fibroblasts, endothelial cells, keratinocytes and inflammatory cells, such as polymorphonuclear leukocytes, lymphocytes and macrophages[47-49] and thus compromise tissue repair. Consequently, HCMV and EBV can weaken defense cells involved in the infectious process and predispose to microbial superinfection.[9]

Once inflamed, periodontal tissues reveal a dense inflammatory infiltrate that contains a wide variety of herpesvirus host cells. HCMV basically infects T lymphocytes, monocytes/macrophages, salivary gland cells, endothelial cells, fibroblasts, polymorphonuclear leukocytes (neutrophils), while EBV infects B lymphocytes and oropharyngeal epithelial cells.[47,48,50,15] HCMV infection, for example, induces a strong antiviral immune response, but it is unable to eradicate the virus from the host. An important characteristic of HCMV is its ability to establish an infection throughout the individual's lifetime.[14]

HCMV exhibits a certain attraction, called cellular tropism, for cells of the immune system, which consequently generates alterations in immunological functions, leading to a state of immunosuppression. Both HCMV and EBV can infect and alter the functions of primordial cells for host defense such as monocytes/macrophages and lymphocytes.[47,48,50,15] According to Kinane et al.,[49] neutrophils are key cells in the control of periodontal infections. HCMV infection can induce abnormalities in adhesion, chemotaxis, phagocytosis, oxidation, secretion and other bactericidal activities of neutrophils.[51] According to a study by Van dyke et al.,[52] defects in neutrophil functions were revealed in localized aggressive periodontitis, which may, in part, be explained by a possible HCMV infection.

Interferences in the innate (nonspecific) and adaptive (specific) mechanisms of the cellular and humoral immune system by HCMV infection can affect *Natural Killer* (NK) cells, deregulate MHC class I and II antigen presentation, in addition to impairing apoptosis.[53] Another factor that unbalances the immune system when the host is infected with HCMV is the fact that this herpesvirus induces the expression of inflammatory mediators such as interleukin 1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α),[54,55] cytokines that are directly involved in the destruction of the periodontium.[56]

Like other members of the Herpesviridae family, EBV can exert important disruptive effects on cytokine synthesis in host cells. Infection of B lymphocytes by EBV can induce changes in lymphocyte populations, favoring the progression of periodontal lesions. Another evident characteristic of EBV is its potential to activate the proliferation of cytotoxic T lymphocytes capable of recognizing and destroying virus-infected cells. EBV infection can even induce the activation of polyclonal B lymphocytes, favoring the production of anti-neutrophil antibodies, and consequently generating neutropenia.[57] EBV-infected B lymphocytes can project antigenic viral structures that result in the production of blocking antibodies and activation of immunosuppressive T cells, just as EBV itself can damage T lymphocyte functions.[54] Similar immunopathological reactions, also associated with infection by other herpesviruses, have been directly linked to the pathogenesis of periodontal diseases in humans.[58]

As observed in the study by Contreras et al.,[8] herpesviruses can be detected in T lymphocytes and monocytes/macrophages in periodontal disease. According to Rouse & Horobov,[59] the infection of defense cells by herpesvirus constitutes an important mechanism of immunity imbalance, especially when infecting T lymphocytes are affected. It is conceivable that the disturbance of the functions of T lymphocytes present in the periodontium may increase tissue destruction.[9] The rapid progression of periodontal disease in HIV-infected patients with decreased T *helper* cell counts suggests the protective importance that T lymphocytes have in periodontitis.[60]

In short, the original sequence described by Contreras & Slots,[9] regarding the development of periodontitis in patients infected by herpesvirus, begins with the infection and activation of the herpesviruses present in the periodontal pockets, according to the compromise of the host's immunological defenses, favoring the growth of pathogenic bacteria, release and alteration of pro-inflammatory chemical mediators, initiation of cytotoxic and/or immunopathological events and consequently destruction of periodontal tissues.

Evidence of HCMV and EBV in periradicular lesions

Periradicular lesions are normally induced as a result of the presence of microorganisms from the oral microbiota in the root canal system,[61] which together with their metabolic products reach the periradicular tissues and induce an inflammatory response.[6,7,62,63] The microbiota of infected canals has been analyzed by several studies presenting a great variance of results, determined not only by the bacterial species present, but also by the method of collection, transport, cultivation and analysis.[63]

According to Sabeti et al.,[12] periradicular lesions are infectious diseases that progress with periods of exacerbation and remission, exhibiting a variety of clinical and radiographic manifestations. Although the involvement of microorganisms and the influence of the host's immune system are unquestionable regarding the development of periradicular pathologies, the pathophysiological events that precede bone destruction or the emergence of an acute inflammation in endodontic pathologies remain undefined.[14]

In periodontics, several studies have investigated the presence of HCMV and EBV in the pathogenesis of periodontal diseases.[9,40,64] Since periodontal infections and endodontic infections share several pathogenic species, studies began to investigate the influence of HCMV and EBV on periradicular pathologies.[12,13,65-68]

Sabeti et al.,[12] carried out a study whose objective was to identify the presence of HCMV, EBV and HSV in periradicular lesions. Seven asymptomatic lesions and 7 symptomatic lesions were collected. According to the results, CMV mRNA was detected in 100% of symptomatic periradicular lesions, while EBV was detected in 6 and HSV was negative in all symptomatic samples. In asymptomatic lesions, the result was positive in only one lesion for each herpesvirus. According to the study data, it can be concluded that HCMV and EBV are closely associated with symptomatic periradicular pathologies. The same study demonstrates that the most extensive lesions presented co-infection of HCMV with EBV.

In a study using five symptomatic teeth, with intact crowns, but with periradicular lesions, we sought to identify the presence of HCMV, EBV and HSV.[65] According to the authors, studies of teeth with intact crowns, but with pulp necrosis, have an advantage over studies of pulp necrosis associated with exposure by carious lesions, as they present pathologies associated with possibly non-infectious causes. The results showed co-infection by HCMV and EBV in 100% of periradicular lesions ranging from 8 X 12 mm to 12 X 15 mm. None of the lesions studied showed active HSV infection.

Sabeti et al.,[66] investigated periradicular infection by HCMV, EBV and HSV in 16 samples from 16 different patients. Fourteen of the 16 samples examined came from teeth that had their canals treated previously and that had a persistent periradicular lesion. Of these 14 lesions, only one was asymptomatic. The two remaining samples were from asymptomatic teeth, which showed no lesion, only the presence of a fractured instrument in the apical region. According to the results, HCMV was identified in 13 of the 16 lesions examined, while EBV was detected in 8. The only asymptomatic lesion was found to be infected by HCMV. Eight lesions revealed co-infection by HCMV and EBV. Only a single symptomatic lesion did not show HCMV or EBV infection. In the two samples referring to the removal of the fractured instrument, the presence of HCMV or EBV was not detected. HSV was not identified in any of the 16 samples examined.

Slots et al.,[68] carried out a study whose objective was to identify HCMV and EBV infection in 44 periradicular lesions (25 symptomatic and 19 asymptomatic). The results revealed that HCMV was present in 100% of symptomatic lesions and in 37% of asymptomatic lesions. Infection with both herpesviruses was reported in 76% of symptomatic lesions and in only 26% of asymptomatic lesions. Alone, HCMV was detected in 24% of symptomatic lesions and in 11% of asymptomatic lesions, while EBV was not observed, in isolation, in any of the 44 lesions. The difference in the occurrence of HCMV and EBV between symptomatic and asymptomatic lesions was statistically significant.

In a study carried out by Sabeti & Slots,[13] we sought to relate the presence of HCMV, EBV and HSV with the clinical characteristics (symptomatic or asymptomatic) and the microbiota prevalent in periradicular lesions. The results of the study revealed that HCMV was present in 22 (96%) symptomatic lesions while 17 (74%) symptomatic lesions were infected with EBV. HCMV and EBV were present in 5 (45%) and 4 (36%) asymptomatic lesions, respectively. Of the total of 34 lesions, 20 (58.8%) were co-infected by HCMV and EBV. *Fusobacterium* species were the most commonly isolated in lesions that presented co-infection with HCMV and EBV, while *Staphylococcus* species were the most frequently isolated in lesions that were negative for HCMV and EBV. HSV infection was observed in only two periradicular lesions.

It has been speculated that herpesviruses can generate periradicular pathologies as a direct result of viral infection and replication or indirectly by damaging host defenses, basically compromising macrophage and lymphocyte functions.[67]

Sabeti & Slots[13] hypothesized that periradicular lesions develop from a series of interactions involving herpesviruses, bacteria and the host's immune system. The authors suggest that an exacerbation of periradicular pathology is attributed to a range of pathophysiological events in which HCMV and EBV play a determining role. Initially, bacterial infection or trauma generates an inflammatory response that promotes reactivation of herpesviruses. Likewise, inflammatory cells, infected by HCMV and EBV are attracted to the region in response to bacterial aggression.

Histopathological features of periradicular inflammatory lesions reveal a large number of cells that can be targets of infection for HCMV and EBV, such as macrophages, T lymphocytes and B lymphocytes.[69] Another striking presence in periradicular lesions are Natural Killer cells[70] and CD8+ T lymphocytes,[71] both characterized as key elements in the host's defense against viral infections.[72]

A significant increase in pro-inflammatory chemical mediators such as interleukin -1β, IL-6, IL-12, tumor necrosis factor- and interferon-γ occurs after HCMV infection.[73] The same occurs when EBV infects the host and triggers an increase in the production of interleukin-1β, antagonist receptor for IL-1 (IL-1Ra), IL-6, IL-8, IL-18, tumor necrosis factor-α and interferon-γ.[53] All these are chemical mediators that are associated with periradicular lesions where they exert a determining potential in bone resorption.[73] This cycle becomes vicious, since the presence of an inflammatory condition is exacerbated by the presence of herpesviruses, and at the same time is one of the factors responsible for the reactivation of HCMV and EBV. The damage caused to the defense cells and consequently the decrease in the resistance of the periradicular tissues favors the multiplication of pathogenic bacteria or possibly cytotoxicity and tissue necrosis.[13]

Sabeti & Slots[13] mention some factors necessary for the development of periradicular pathology, highlighting the importance of occurring simultaneously: presence of herpesviruses in the periradicular region, activation of latent viruses, inadequate anti-viral response of cytotoxic T lymphocytes, presence of pathogenic microorganisms and insufficient levels of antibacterial antibodies.

According to the authors, in this way the factors would be cooperating with each other in a destructive cascade, infrequent and according to the patient's periods of immunosuppression.

Among other pathogenic factors attributed to herpesvirus infection, it is known that HCMV and EBV affect the humoral immune response, which strives to produce antibodies against surface protein molecules seeking to inactivate the virus, in addition to compromising the cellular immune response. that constitutes the specific defense against herpesvirus infection and that tries to eliminate infected cells through cytotoxic CD8+ T lymphocytes that recognize viral peptides on the surface of infected cells expressed by the major histocompatibility complex of class I and II (MHC I and MHC II).[53]

Prevalence of HCMV and EBV in immunocompromised and immunocompetent individuals

HCMV is an opportunistic pathogen that can generate severe pathologies during the course of life, especially in patients with HIV.[34,74] According to Peterson et al.,[75] HCMV plays an important role in the transmission and progression of HIV through a series of mechanisms that can explain the adverse effects of a co-infection. HCMV could activate HIV directly or indirectly, increasing the production of cytokines that would allow HIV to infect cells that were previously resistant to infection. Several severe diseases have been reported exclusively in patients infected simultaneously by both viruses.[76-79]

Epidemiological studies involving HIV-positive or HIV-negative patients demonstrate that HCMV is present in a large part of the world's population. In comparative studies, it has been reported that the prevalence of HCMV in HIV-positive patients is higher[80,81] or similar[82,83] to control groups with healthy patients.

For a period of 23 months, Gerberding[84] evaluated 976 health professionals in San Francisco, United States, regarding infection by several viruses, including HIV and HCMV. The results revealed that HIV was not detected in any professional, while 43% of the individuals tested were positive for HCMV.

As it is an infection that persists throughout life, 60 to 100% of individuals who reach the age of majority are seropositive for HCMV, depending only on social and geographical factors, a fact even more evident in HIV-positive patients.[85]

In 1996, EL-NAWAWY et al.[86] carried out a study in a rural area of Egypt whose objective was to determine the prevalence of HCMV in recent mothers. The results revealed that 143 mothers (96%) tested positive for HCMV.

Badalti et al.,[87] investigated the prevalence of HCMV infection in HIV-positive patients. The results revealed that 100% of the patients had HCMV infection. In order to elucidate the prevalence of HCMV in HIV-infected patients in France, Robain et al.,[88] analyzed 1504 patients. The results revealed that HCMV was detected in 87% of the individuals, mainly in homosexual men.

In a study carried out in Rio de Janeiro, Yamamoto et al.,[89] evaluated the incidence of HCMV in 189 newborns and their mothers. According to the results, HCMV was detected in 95% of mothers in contrast to only 2.6% of newborns. Lu et al.,[90] analyzed blood samples from 1800 blood donors in Taiwan, seeking data on the prevalence of HCMV in the population. The results revealed that only 8% of the donors had a negative result.

HCMV is a virus common to all places, which varies from 40 to 60% of seropositivity in adults in Western countries. Congenital infection occurs between 0.15 to 2% of newborns, but the most worrying thing is that 30% of these develop active disease such as mental retardation, while 18% develop long-term sequelae such as hearing loss. In childhood, 60% of children between 1 and 2 years of age are infected with HCMV, but rarely develop any pathology.[91]

Pultoo et al.,[92] demonstrated a high incidence of HCMV infection in an Indian population. Of the 584 volunteers for the blood test, HCMV was detected in 93% of patients.

In the city of São Paulo, Cunha et al.,[74] used the PCR method to assess the incidence of HCMV in HIV-positive patients. Of the 237 individuals evaluated, 34% tested positive for HCMV. However, the authors state that the findings were strongly influenced by the impact of antiretroviral therapy, which significantly reduces the incidence of HCMV viremia, as well as the occurrence of active disease in HIV-infected patients. For Kaplan et al.,[93] although the use of antiretroviral therapy has led to a considerable decline in the incidence of opportunistic infections in AIDS, individuals with advanced disease are susceptible to developing diseases related to HCMV infection. HCMV-associated disease in HIV-infected patients is usually the result of reactivation of a pre-existing latent infection. In HIV-positive patients, HCMV-associated diseases commonly manifest when the CD4+ T lymphocyte count is below 100 cells/mm.[94]

In Nyumbani, Kenya, Chakraborty et al.,[95] tested 71 HIV-infected children, seeking to investigate a possible co-infection with HCMV. The results revealed that all selected children were also infected with HCMV. Recently in Madrid, Spain, Ory et al.,[96] carried out a study comparing the prevalence of HCMV in healthy women in 1993 and 1999. Age was a determining factor for the separation of the studied groups (2-5, 6-10, 11-15, 16-20, 21- 30 and 31-40 years old). According to the results, seropositivity was low for all groups and without statistical differences, except in the groups aged 6-10 years, where the incidence increased from 44% to 57%, and 31-40 years, where the prevalence also increased from 79. % to 90%.

Several evidences suggest that EBV is commonly found in standard tests performed in individuals with AIDS or who are at high risk of developing such a disease.[23,97] Apparently, the EBV that remains latent in immunocompetent individuals tends to reactivate in immunocompromised patients, inducing the emergence of several diseases as well as greater immunosuppression.[98,99]

Opportunistic viruses, especially EBV, are frequently considered pathological agents in the oral lesions of HIV-positive patients. In the literature, several studies confirm the presence and/or influence of EBV in pathologies characteristic of patients with HIV.[100-102] Sculley et al.,[103] performed a study seeking to identify EBV type 1 and 2 in lymphocytes from 26 HIV-positive patients. The results presented revealed that in 100% of the cases EBV was detected. Type 1 was reported in 69% of samples, while type 2 in 19% and co-infection with types 1 and 2 in 12%. In the same study, the authors also highlighted that EBV type 2 is more commonly present in HIV-positive patients than in healthy patients.

Peripheral lymphocytes obtained from 94 EBV-infected individuals, in addition to another 33 samples collected from gargling solutions from healthy people, were analyzed by Apolloni & Sculley.[104] Specific primers were used for both types of EBV (EBV-1, and EBV-2). The results revealed that in 78 samples (83%) EBV was detected. Of the positive samples, EBV-1 was detected in 35%, EBV-2 in 21%, and both in 27%. Of the samples from healthy patients analyzed, 21 (64%) revealed the presence of EBV, of which type 1 was present in 11 (52%), type 2 in 7 (33%), and both in the 3 remaining samples (14 %).

According to Weiss,[21] more than 90% of the world population is infected by EBV, but only about 1% of individuals develop pathologies related to EBV, such as lymphomas and nasopharyngeal carcinomas. The same author also emphasizes that immunocompromised individuals, especially those who already show symptoms of AIDS, are the most likely to develop types of cancer related to virus infection, which makes patients more susceptible to multiple infections.

Aiming to find new data on the pathogenesis of some viruses of the *Herpesviridae* family in ocular pathologies of HIV-positive patients, Lee et al.,[105] collected conjunctival microfragments from 30 HIV-positive patients and also from 30 HIV-negative patients. PCR was used to detect the presence of EBV and HCMV DNA among other viruses. HCMV was detected in 20% (6 of 30) of the HIV-positive patients and in none of the HIV-negative patients. EBV was detected in 40% (12 of 30) of HIV-positive patients and in 47% (14 of 30) of HIV-negative patients. The same study also highlighted the high incidence of infection by the viruses of the *Herpesviridae* family, both in HIV-positive and HIV-negative patients.

Baarle et al.,[106] investigated the prevalence of EBV type 2 in males according to their sexual orientation (homosexual **o**r heterosexual). The authors selected 85 HIV-positive homosexual patients (group 1), 113 HIV-negative homosexual patients (group 2), and 114 HIV-negative heterosexual patients (group 3). According to the results, EBV could be detected in 97% of patients in group 1, 80% of group 2, and in 82% of group 3. According to the authors, the difference in EBV infection among homosexual patients and heterosexuals was significant, however they emphasized that the data reveal that the general population has high rates of contamination by EBV type 2.

Ikuta et al.,[107] analyzed the prevalence of EBV in saliva and in a gargle solution of healthy subjects in Japan. According to the results, viral DNA was reported in 90% of the 48 gargle samples and in 38% of the 93 saliva samples. Aiming to determine the presence of EBV DNA, among other viruses, in the oral mucosa of humans, Ammatuna et al.,[108] selected 57 HIV-positive subjects and 30 healthy subjects as controls. The results presented showed that EBV was detected in 42% and 16% of samples from HIV-positive and healthy patients, respectively. The authors concluded that the EBV genome was significantly detected in the oral mucosa of HIV-positive patients, which would explain the strong link between HIV and EBV co-infection with the appearance of hairy leukoplakia.

Ozkan et al.,[109] investigated EBV seropositivity in 540 people from Elazig, Turkey. The results revealed that 99% of the individuals studied were infected with EBV.

Proposition

The objective of this study is to compare the incidence of HCMV and EBV in asymptomatic periradicular inflammatory lesions of HIV-positive and HIV-negative patients using the immunohistochemical method.

Materials and methods

Collection of periradicular specimens

Asymptomatic primary periradicular lesion samples were collected at the time of previously scheduled tooth extraction surgery, where each patient became aware of the study and signed a consent form. The teeth showed carious lesions and absence of periodontal pockets more than 4 mm deep. The reason for the extractions was prosthetic. Eighteen patients were included in the study, nine of whom were diagnosed as HIV-positive based on ELISA tests and confirmed by *Western Blot*.

Prior to the administration of local anesthesia, the tooth, gum and oral mucosa were decontaminated with 0.12% chlorhexidine solution. Then, the patients were instructed to rinse their mouths with the same solution for 30 seconds. After extraction, the tooth, together with the lesion attached to the root apex, was washed with sterile saline and the lesion was removed with the aid of a #15 sterile scalpel blade. Eighteen lesions (09 from HIV-positive patients and 09 from HIV-negative patients) were then immediately fixed in 10% buffered formalin and embedded in paraffin. Specimens were analyzed by histological staining for immunohistochemical classification for further investigation of herpesvirus-infected host cells.

The lesions were submitted to serial histological sections and stained with hematoxylin-eosin and Gomori's trichrome. The sections were then analyzed using an optical 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). from Media Cybernetics).

Immunohistochemical analysis

Immunohistochemical procedures were performed using a standard protocol based on the streptavidin-biotin complex technique. After deparaffinization and hydration, the sections were immersed in 0.01mM/pH=6.0 of citrate buffer (PRQ Química Ltda – C050K) in a water bath at 95ºC for 40 min to preserve the antigens for later immunostaining. The specimens were then treated for 20 minutes with 3% H_2O_2 in methanol at room temperature to neutralize the effects of endogenous peroxidase, then washed with phosphate-buffered saline (PBS) pH=7.6 at room temperature. Subsequently, the blocking of non-specific binding was performed with goat serum for 10 minutes at room temperature and the slides were then incubated overnight in a humid chamber with the primary antibodies (Table 1): mouse monoclonal anti-Cytomegalovirus antibody human (Dako–M0854, final dilution 1:50, Golstrup, Denmark) and anti-Epstein-Barr Virus (Dako–M0897, final dilution 1:250), followed by incubation with secondary biotinized antibody for 15 minutes and with the streptavidin complex -biotin for another 15 minutes (Universal DakoCytomation LSAB® + System HRP, Dako–K0690).

Table 1 Primary antibodies used for immunohistochemical staining of periradicular lesions

Antibody	Type	Reactivity	Dilution
Human Cytomegalovirus M0854	Camundongo Monoclonal/DDG9	76 kDa ptn	1:50
	Camundongo Monoclonal/CCH2	DNA-binding ptn (p52)	
Epstein-Barr virus LMP M0897	Camundongo Monoclonal/CS1-4	60 kDa LMP*	1:250

*Latent protein membrane –1

The peroxidase reaction was detected with the chromogenic substrate solution system containing 3,3'-diaminobenzidine and hydrogen peroxide (DakoCytomation Liquid DAB + Chromogenic Substrate System, Dako–K3468).

Finally, the sections were stained with Harris' hematoxylin and mounted in synthetic resin. All washing steps were performed with phosphate-buffered saline (pH=7.6) at room temperature. Positive controls were performed using histological sections of human lymphomas with the participation of both viruses.

The DDG9 clone anti-human Cytomegalovirus antibody reacts with the 76 kDa protein, while the CCH2 clone reacts with the p52

protein. None of the antibodies react with structural HCMV antigens nor do they cross-react with other herpesviruses. The reaction occurs with HCMV-infected cells providing a nuclear staining pattern. Furthermore, at a late stage of the infection, a cytoplasmic positivity reaction can also be detected. The anti-Epstein-Barr virus antibody reacts with the latent membrane protein-160 kDa (LMP) encoded by the EBV gene BNLF1. All 4 anti-LMP antibodies present in the product recognize distinct epitopes in the hydrophilic carboxyl region of the LMP. All immunostaining were performed in triplicate.

Statistical analysis

Comparisons between the number of HCMV and EBV-infected lesions in HIV-positive or HIV-negative patients were performed using Fischer's exact test. The significance level was determined at $p \leq 0.05$.

Results

The results showed that of the 9 lesions collected from HIV-positive patients, 67% were infected with HCMV while 44% were infected with EBV (Table 2). In HIV-negative patients, HCMV and EBV were detected in only 11% of the samples. Co-infection between HCMV and EBV was demonstrated in 33% and 11% of lesions from HIV-positive and HIV-negative patients, respectively.

Table 2 HCMV and EBV infection data in periradicular lesions

Periradicular Lesions	HCMV infection	EBV infection	Co-infection (HCMV+EBV)	Absence of infection
HIV-positive (n=9)	6* (67%)	4 (44%)	3 (33%)	2 (22%)
HIV-negative (n=9)	1 (11%)	1 (11%)	1 (11%)	8 (89%)

*Number and percentage of positive samples.

Statistical analysis revealed that the incidence of lesions containing HCMV-infected cells was significantly higher in HIV-positive than in HIV-negative patients (p=0.02). There was no significant difference when EBV data were evaluated (p=0.15), even though the number of lesions infected by this virus was 4 times higher in HIV-positive patients.

Discussion

For many years, acute episodes in cases of primary endodontic infection or even in asymptomatic chronic pathologies have been associated almost exclusively with bacterial infection and the immune status of the host. However, some treatments performed even inappropriately can provide the repair of periradicular tissues.[89] Thus, determining the factors that lead to success or failure in endodontic treatment still remains uncertain, requiring further clarification.

The main finding of this study concerns the identification of HCMV infection in 6 of 9 asymptomatic lesions examined from HIV-positive patients when compared to HIV-negative patients. Although the incidence of EBV infection was higher in HIV-positive patients, the difference was not statistically significant when compared to HIV-negative patients. It is possible that the lack of statistical significance is related to the sample size, which reinforces the need for further studies using a larger sample, which may better elucidate this fact.

Co-infection was detected in 33% of HIV-positive patients and in 11% of healthy patients. These results are in agreement with the findings of Slots et al.,[14] where they identified co-infection by HCMV and EBV in 26% of asymptomatic samples, and data from Sabeti & Slots[13] who reported co-infection in 36% of asymptomatic samples.

The present study even demonstrates a higher incidence of herpesvirus, especially HCMV, in HIV-positive patients, when compared to HIV-negative patients. Although pioneering in terms of studies on periradicular lesions, these data are in agreement with other studies in the literature that demonstrate a high incidence of HCMV infection in HIV-positive patients.[80,81,87] The occurrence of HCMV and EBV in low incidence in asymptomatic lesions of healthy patients confirms the findings of Sabeti et al.[12]

The results of the analysis of lesions from HIV-positive patients conflict with the findings of Sabeti et al.[12], since in a sample of 7 asymptomatic lesions, HCMV and EBV were detected in only one sample each, whereas in the present study, HCMV and EBV were reported in 6 and 4 samples respectively from a total of 9 asymptomatic samples. Another result conflict involving the lesions of immunocompromised patients used in this study can also be seen in the work by Slots et al.,[14] in which the authors did not detect EBV in any asymptomatic sample, but there was a consensus regarding the detection of HCMV in 7 of the 19 samples. The results of this study are consistent with the findings of Sabeti & Slots,[13] where they analyzed 11 asymptomatic samples identifying HCMV and EBV in 5 and 4 samples, respectively.

To date, no study has evaluated the incidence of HCMV and EBV in symptomatic or asymptomatic periradicular lesions of HIV-positive patients. This brings up the need to diagnose any immunosuppressive disease that may somehow induce results. Several studies point to a higher incidence of herpesvirus infection in HIV-positive patients when compared to the healthy control group.[80,81] The same occurred in the study by Contreras et al.,[42] who identified a higher incidence of herpesvirus in the periodontal disease of HIV-positive patients than when compared with healthy patients.

Studies report a high positivity of HCMV infection, ranging from 87%[88] to 100%[87] in HIV-positive patients. Also a common factor regarding EBV infections.[23,103] Although epidemiological studies involving healthy patients point to a high incidence of HCMV and EBV infection,[29,110] individuals considered in the risk group are the most susceptible to herpesvirus infections, as well as HIV itself.[7,15]

Symptomatic periradicular lesions are relatively uncommon, perhaps because a series of pathophysiological events is necessary to establish an acute endodontic infection. There is a lot of evidence implicating HCMV and EBV as cofactors or triggering agents in the etiopathogenesis of periradicular lesions. As seen in several studies evaluating the presence of herpesvirus in periradicular lesions, HCMV and EBV have a high incidence in symptomatic lesions,[78-81,94] which, however, were not examined in this study. Although the incidence of asymptomatic lesions was less frequent, it must be considered that such asymptomatic lesions may, in some cases, precede symptomatic ones.

The infection and consequent activation of herpesviruses in periradicular tissues can induce significant immunosuppression and alterations in the host's local defenses.[94] This may favor the multiplication of Gram-negative anaerobic bacteria, whose structural products can stimulate a greater production of chemical mediators by the host cells,[81] inducing a greater inflammatory response and destruction of bone tissue. In fact, the herpesvirus-bacteria interaction appears to be bidirectional, since bacterial by-products and inflammatory chemical mediators have the potential to activate local herpesviruses (vicious cycle concept).[110]

It is extremely important to pay attention to the hypothesis that acute episodes develop preferentially in immunologically immature or immunocompromised individuals, who are unable to establish an

adequate defense against infectious agents, a fact that would be even more evident in patients infected with HCMV and EBV.

Since periradicular inflammation resulting from pulp necrosis and infection constitutes the initial event that generates the development of periradicular disease in the current herpesvirus-bacteria-immune system model, the diagnosis and histopathological implications of the present findings need to be better clarified. It seems quite reasonable to consider periradicular lesions as one of the infectious diseases in the list of those influenced by both herpesviruses, particularly in HIV-positive patients, due to the close association found between HCMV and EBV with lesions in such patients.

Conclusion

The results of the present study confirmed that periradicular lesions can be infected by herpesvirus. The incidence of HCMV infection was significantly higher in HIV-positive than in HIV-negative patients. The role of herpesviruses in the pathogenesis of periradicular lesions needs to be better elucidated.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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