Human Cytomegalovirus and Epstein–Barr Virus in Apical and Marginal Periodontitis: A Role in Pathology?

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Periodontitis is presumably caused by bacterial infection, but it has been shown recently that affected tissue often contains human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV). The present study was initiated to evaluate the role of these viruses in the pathogenesis of periodontitis. HCMV and EBV were quantified in 40 apical and 25 marginal periodontitis samples using real time PCR. In situ hybridization or immunohistochemistry was carried out on apical samples to detect viral presence within cells. A possible association with relevant bacteria was examined. Of the apical periodontitis samples, 50% contained EBV, while none contained HCMV. Of the marginal periodontitis samples, 40% were positive for EBV and 12% for HCMV. With one exception, however, the amount of virus was close to the detection limits. EBV was only detected in 1 out of 15 healthy periodontium samples. Immunohistochemistry and in situ hybridization were all negative. Significant associations were found between periodontal EBV and the presence of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. Although there was an obvious association of the virus with clinical samples, it seems unlikely that these viruses play a major role in the pathogenesis of periodontitis of the average patient. Their presence may reflect that the clinical samples contain more blood or saliva compared to controls, or an accumulation of lymphoid cells harboring virus in the inflamed tissue. *J. Med. Virol. 80:1007–1011, 2008.*

KEY WORDS: Epstein–Barr virus; cytomegalovirus; apical periodontitis; marginal periodontitis; quantitative PCR; in situ hybridization

INTRODUCTION

It is well documented that apical and marginal periodontitis are associated with bacteria colonizing the root/tooth surfaces [Kakehaski et al., 1965; Socransky and Haffajee, 2002]. Recently, however, it has been shown that these lesions often contain viruses of the herpes family, particularly human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) [for reviews see Slots, 2004; Cappuyns et al., 2005; Slots et al., 2006]. Virus and bacteria may act in synergy to produce pathology. Slots et al. [2006] suggested that herpesviruses can influence the development and course of periodontitis, while Cappuyns et al. [2005] are skeptical.

Both EBV and HCMV are present latently in the vast majority of the adult population. EBV is primarily harbored in B cells and in epithelial cells of the oropharynx, while HCMV is present in a greater variety of cells including various epithelial cells, endothelical cells, and leukocytes. Both viruses are active periodically and shed viral particles to the saliva.

Active replication in periodontal tissues can be envisaged easily to impact the immune response in a way that benefits both opportunistic bacteria and the virus, and thus lead to aggravated symptoms. For example, the viruses produce cytokine mimics designed to modulate immune defense. On the other hand, the presence of virus in affected tissues does not prove an active role, it may just reflect normal viral replication. It should also be noted that microbial activity can induce

viral replication, as has been shown recently in the case of EBV and malaria [Chêne et al., 2007].

If the impact of viral replication on the bacterial environment is real, then it might be expected that the bacterial profile differs between sites with or without virus. Certain correlations of this sort have been reported [Contreras et al., 1999; Michalowicz et al., 2000; Ting et al., 2000]. The bacterial profiles of marginal periodontitis samples were therefore investigated.

As EBV and HCMV may be present in both saliva and blood, care was taken to avoid unnecessary contamination. In the case of apical periodontitis, samples are easily contaminated with microorganisms present in the marginal area [Sunde et al., 2000]. Previously, samples have been obtained typically by marginal incisions [Slots et al., 2003]. In order to investigate this source of contamination, samples were obtained by either a marginal or submarginal incision.

The present study was initiated in an attempt to obtain more data relevant to evaluating the role of HMCV and EBV in periodontal disease. Samples from chronic marginal periodontitis and refractory apical periodontitis, the latter in teeth not responding to conventional root-canal therapy, were obtained; along with samples from healthy periodontium. The first objective was to estimate the prevalence, and quantify the amount of virus in Norwegian samples. Most previous reports are from North America, and most are based on non-quantitative PCR methods. Thin sections obtained from apical samples were investigated further with in situ hybridization or histocytochemistry in order to detect viral activity and/or the presence within cells. Local replication would indicate a clinical role.

MATERIALS AND METHODS

Patients

Twenty-five patients (age 37–75, mean age 56) with "refractory" marginal periodontitis, based on clinical and radiographic findings [Armitage, 1999], were recruited. All periodontal pockets probed were ≥6 mm. All patients had been treated with conventional periodontal therapy before sampling. After isolation and drying of the area to be sampled with cotton rolls, subgingival plaque specimens were removed carefully by a sterile curette. None of the patients had systemic diseases or had taken antibiotics during the last 2 months before sampling. These patients were compared with 15 healthy volunteers (30–67, mean age 45) with no attachment loss or gingival bleeding on probing. After isolation and drying with cotton rolls, three sterile paper points were inserted to the depth of the selected gingival sulcus and left in place for 30 sec.

Another 40 patients (age 27–93, mean age 56) with apical periodontitis on therapy resistant teeth were surgically treated with apicectomies by the same oral surgeon. Approximately half the teeth (18) were considered symptomatic, that is, apical tenderness to palpation

and/or apical tenderness. The remaining 22 cases were considered asymptomatic. No patients had pain at the time of surgery. In half of the patients, a marginal incision was made, while in the other half a submarginal incision was applied in order to avoid contamination of the periapical lesion with microorganisms present in the marginal area.

The samples obtained with a submarginal incision were divided in two with a sterile scalpel. Half was put in 4% formaldehyde for in situ hybridization and immuno-histochemistry, the other half was transferred to 200 μl of lysis buffer from the extraction kit (QiaAmp® DNA Mini Kit, Qiagen Crawley, West Sussex, UK) and subsequently kept at −20°C prior to extraction. The extraction was according to the manufacturer's recommendation, using 50 μl elution buffer for marginal samples and 200 μl for apical samples.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK). Informed consent was received from all patients and volunteers.

Real Time PCR

Viral DNA was tested using commercial real time PCR kits according to manufacturer's instruction (ReSSQ CMV and ReSSQ EBV based on LightUp Technologies and supplied by Dako Norden AS, Denmark). Five microliters of extract was included in the PCR reaction. Negative samples and supplied standards were included in each run for accurate quantitation. Numerical quantification was achieved with the help of the program ViroQalc™. The detection limits of the assays, according to the manufacturer, were respectively 4.2 and 1.4 copies per μl in the case of EBV and HCMV. The dynamic ranges for quantitation were respectively 10^2–10^8 and 10–10^8 per reaction. An extraction control DNA (included in the kit) was added to each sample prior to extraction. An independent PCR based on this DNA was performed for each sample, verifying both that DNA was not lost during extraction, and that the extract did not contain inhibitory substances. The PCRs were run on a LightCycler (Roche, Indianapolis, IN). The amount of virus present in the actual oral samples was calculated.

In Situ Hybridization and Immunohistochemistry

In situ hybridization for EBV was performed on the 20 apical lesions obtained by submarginal incision. The same samples were also examined for HCMV using immunohistochemistry. Formalin-fixed biopsy material was embedded in paraffin, 2–4 μm thick paraffin sections were cut, placed on precoated (poly-L-lysine) object glasses, and dewaxed. One section from each biopsy was stained with hematoxylin-eosin for morphological assessment. For the detection of EBV, a cocktail of oligonucleotide probes specific for EBV early RNA (Ventana Medical Systems, Illkirch, France, catalogue # 780-2842) was applied. The hybridization was performed

in a Ventana XT autostainer. Pretreatment of slides and hybridization conditions were adjusted according to the supplier's recommendations. Detection of hybridized probes was done with the iVIEW Blue Plus kit from Ventana, resulting in a blue reaction product localized to EBV-infected nuclei. For positive control, a section from an EBV-positive lymphoproliferation was placed on each slide beside the section from the apical biopsy. Sections were counterstained with Kernechtrot and evaluated in a light microscope.

For immunohistochemical detection of CMV, a mouse monoclonal antibody against the early DNA-binding CMV protein p52 (DAKO, DK-2600 Glostrup, Denmark, code no. M 757) was applied. Pretreatment of slides and the immunohistochemical procedure were performed in a Ventana XT autostainer, and positive reactions were visualized with the iVIEW DAB kit providing a brown reaction product in infected nuclei. For positive control, a section from CMV-infected colon mucosa was included on each slide. Sections were counterstained with hematoxylin and evaluated in a light microscope.

Bacterial Classification

The 25 marginal periodontitis samples were tested individually for the presence of 20 bacterial species using checkerboard DNA–DNA hybridization [Socransky et al., 1994], with modification described by Haffajee et al. [1997]. Briefly, 0.15 ml of 0.5 M NaOH was added to 0.15 ml DNA extract. DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes for 20 subgingival species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using alkaline phosphatase-conjugated antibody to digoxigenin and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL), and were read using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA). Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were converted to absolute counts by comparison with the standards on the same membrane.

RESULTS

In the case of marginal periodontitis, 10 of the 25 samples contained EBV, while only 3 contained HCMV (Table I). Only 1 out of 15 samples from healthy teeth had a barely detectable presence of EBV, and none contained HCMV. With one exception, all samples had low levels of virus. The one exception, having

TABLE I. Prevalence and Quantity of Virus in Patients With Marginal Periodontitis (N = 25) Compared to Healthy Controls (N = 15)

Sample	EBV	HCMV
Marginal periodontitis (N = 25)	10 (40%)[a]	3 (12%)
Range of viral copies	200–8,000, 353,000[b]	120–2,400
Average	37,060	900
Healthy periodontium (N = 15)	1 (7%)[a]	0
Range of viral copies	35	
Average	35	

The samples were analyzed using real time PCR, and the actual number of viral genomes in the complete sample calculated.
[a]$P = 0.03$ using the Chi-square test (Fisher's Exact-Exact Sig. 2-sided).
[b]The high number of copies was detected in a single patient.

350,000 copies of virus, was a case with an untreatable and particularly troublesome marginal periodontitis.

In teeth with apical periodontitis, 13 of the 18 symptomatic teeth samples contained EBV, while only 7 of the 22 asymptomatic teeth samples (Table II). None of the samples contained HCMV. When the same data were divided as to whether the incision was made marginally or submarginally, it was detected virus more often using a marginal incision, although the difference was not statistically significant ($P = 0.056$, Fisher's Exact test). None of the samples contained appreciable amounts of virus.

No signs of cells harboring virus were found in the 20 apical samples analyzed by in situ hybridization for EBV and immunohistochemistry for HCMV.

The marginal periodontitis samples were investigated, using DNA hybridization, for the presence of the 20 types of bacteria considered the more relevant in connection with periodontitis. The number of species per lesion was

TABLE II. Prevalence and Quantity of Virus in Patients (N = 40) With Symptomatic or Asymptomatic Apical Periodontitis

Sample	EBV	HCMV
Symptomatic patients (N = 18)	13 (72%)[a]	0
Range of viral copies	50–900	
Average	300	
Asymptomatic patients (N = 22)	7 (28%)[a]	0
Range of viral copies	50–4,000	
Average	970	
Marginal incision (N = 20)	13 (65%)[b]	0
Range of viral copies	50–4,000	
Average	588	
Submarginal incision (N = 20)	7 (35%)[b]	0
Range of viral copies	50–817	
Average	378	
Infected cells	None detected	None detected

The samples were obtained by either marginal or submarginal incisions. Viral load was measured with real time PCR, and the actual number of viral genomes in the complete sample calculated. Samples obtained by submarginal incision were also analyzed for infected cells by in situ hybridization for EBV and immunohistochemistry for HCMV.
[a]$P = 0.025$ and [b]$P = 0.056$ using the Chi-square test (Fisher's Exact-Exact Sig. 2-sided).

7–18 (mean 13.3). Species most often detected were *Fusobacterium nucleatum* ssp. *vincentii* and *Streptococcus intermedius*, present in 23 of 25 samples (92%). *Treponema denticola* and *Tannerella forsythia* were present in 17 of 25 samples investigated (68%). *Porphyromonas gingivalis* was found in 12 of 25 samples (48%). *A. actinomycetemcomitans* was detected in 8 of 25 samples (32%).

The presence of virus did not appear to influence the bacterial flora, with two possible exceptions: associations were observed between EBV and respectively *P. gingivalis* (present in 80% of EBV positive samples while only 27% of negative samples, $P = 0.015$ in Fisher's Exact test); and *A. actinomycetemcomitans* (present in 60% of EBV positive samples while only 13% of negative samples, $P = 0.028$ in Fisher's Exact test).

DISCUSSION

The present results confirmed previous reports associating EBV with marginal and apical periodontitis. HCMV, however, was not found at all in the apical samples, and only rarely in the marginal samples. The low prevalence of HCMV is in line with results reported by Klemenc et al. [2005], while Slots [2004] has reported considerably higher prevalence.

Most previous reports used non-quantitative PCRs for viral detection. In the present study, real time PCRs were performed, assuming that the amount of virus is an important parameter when evaluating a role in pathogenesis. The point is particularly relevant as both EBV and HCMV replicate in cells associated with the oral cavity, and their presence in saliva and oral tissue is well documented [Sixbey et al., 1984; Rickinson and Kieff, 2001; Roizman and Pellett, 2001]. The amount of virus was, with one notable exception, found to be close to the detection limits.

The marginal samples were obtained from either curette, in the case of clinical samples, or paper points, in the case of controls. Regardless of sampling tools, more material is expected to be obtained from a periodontal pocket compared to a healthy pocket. The number of lymphocytes, which may carry latent viruses, is significantly higher in sites with active periodontitis compared with stable sites [Zappa et al., 1991]. The observation (Table I) that only one of the controls contained virus should be considered with these limitations in mind. In other words, as most positive samples were close to the detection limit, it is difficult to interpret whether the increased prevalence in affected tissue was due to sampling bias or to an actual activation of viral replication.

As to apical periodontitis, HCMV was not identified while EBV was detected in 45% of the lesions, which is less than in some previous studies [Sabeti et al., 2003; Slots et al., 2003]. The sensitivity of the PCR methods may influence the results, but so may the surgical technique. The samples are easily contaminated with microorganisms from the marginal area unless care is taken to avoid such contamination [Sunde et al., 2000]. As shown in Table II, the prevalence of EBV decreased appreciably when performing a submarginal incision in order to avoid marginal contamination. In line with previous investigations [Contreras and Slots, 1998; Ting et al., 2000; Sabeti et al., 2003], Table II also reflects that virus was found more often in symptomatic compared to asymptomatic apical periodontitis.

Several studies have quantified the levels of EBV and HCMV in blood from patients with various virus associated clinical symptoms [Cohen, 2005; Gouarin et al., 2007; Hakim et al., 2007]. It should be noted that even in these cases, the viral loads differ drastically. Thus one cannot exclude that even a low level of virus may be relevant clinically. It is, however, assumed that in order to have an impact, the viruses are required to be active.

Viral activity was examined in 20 apical samples using respectively in situ hybridization (for EBV) and immunohistochemistry (for HCMV). As a single viral genome can produce a large number of transcripts and proteins, the detection of these products can actually be more sensitive than genomic PCR. No signs of viral activity were demonstrated, suggesting that the viral genomes detected in the apical samples may reflect either free viral particles or cells containing dormant viruses.

It should be noted that the levels of EBV and HCMV in saliva appear to be associated with periodontal status as well, that is, the levels are increased in affected individuals, and decrease upon treatment of the condition [Idesawa et al., 2004; Saygun et al., 2005].

Evidence is accumulating that viruses may play a role in several conditions previously thought to be solely of bacterial origin, including otitis media and respiratory tract infections [Bakaletz, 1995; Heikkinen and Chonmaitree, 2000; Cappuyns et al., 2005]. In the case of herpes viruses, even low levels of viral activity can theoretically aggravate bacterial infections, for example by the release of cytokine mimics. Herpesviral–bacterial interactions can help explain various clinical characteristics of marginal and periapical infections: The alteration between prolonged periods of virus latency interrupted by periods of activation may be responsible for the typical flare-ups observed in patients with periodontal problems.

EBV and HCMV have been reported to correlate with the presence of specific periodontopathic bacteria, which would be expected if the viruses impact on the bacterial environment. In a study of gingivitis and periodontitis, virus was associated with an increased occurrence of *P. gingivalis*, *T. forsythia*, *P. intermedia*, *P. nigrescens*, and *T. denticola* [Contreras et al., 1999]. Another study compared samples from two disease-active and two disease-stable periodontitis sites in each of 16 patients [Kamma et al., 2001]. They found that EBV and HCMV were associated with *Dialister pneumosintes* and *P. gingivalis*, as well as with the disease-active sites. HCMV-associated localized juvenile periodontitis lesions also exhibit elevated levels of *P. gingivalis*

[Michalowicz et al., 2000] and *A. actinomycetem-comitans* [Contreras et al., 1999; Ting et al., 2000]. The present study found significant associations between EBV and the latter two bacterial species, but not the other 18 species tested, which included the other species mentioned above with the exception of *D. pneumosintes*.

Both the bacterial and the other associations described in the above paragraphs may be taken to suggest an active role of virus in the pathogenesis. Alternatively, they could simply reflect the severity of the condition and degree of inflammation. A more active inflammation would be expected to correspond to the presence of particular bacteria, cause more pain, contain more lymphoid cells harboring virus, and/or more bodily fluids containing virus.

It is becoming more and more obvious that a variety of viruses can be present and replicate in the human body without causing any symptoms. Care is therefore required when considering the clinical relevance of an association between virus and a pathological condition. Even in cases where there is a significant overrepresentation of virus in clinical samples compared to healthy controls, the association may be due to confounding factors. For example, circoviruses have been considered as a cause of hepatitis and as a cofactor in AIDS, but a more likely explanation is simply that an impaired immune status causes an increase in viremia [reviewed by Grinde, 2003].

Although a role of EBV and HCMV in the etiopathogenesis of periodontal morbidity cannot be excluded, the present data suggest that in the limited samples studied, these viruses did, in most of the cases, not have any major effect. In order to influence the etiology, one might expect local viral activity. Thus either a high concentration of virus, or the detection of cells producing viral RNA or proteins, would suggest a more prominent role.

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