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Low prevalence of subgingival viruses in periodontitis patients

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Abstract

Background: Viruses such as Human Cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) have been proposed to be periodontal pathogens. The aim of this study was to analyse the presence of herpesvirus DNA in subgingival plaque samples of patients with different forms of periodontitis and in healthy periodontia.

Materials and Methods: A total of 140 ethnically mixed (prevalently Caucasian) subjects took part in the study. Sixteen were affected by localized aggressive periodontitis (LAgP), 64 by generalized aggressive periodontitis (GAgP), 20 by chronic periodontitis (CP) and 40 were periodontally healthy. Polymerase chain reaction (PCR) analyses were performed to detect HCMV and EBV. Sera were tested for anti-HCMV and EBV IgG antibodies. PCRs for herpes simplex (HSV) and varicella zoster virus (VZV) were performed in subgingival samples from a subset of 20 AgP subjects.

Results: HCMV DNA was not detected in any plaque samples. EBV DNA was detected in four LAgP (25%), two GAgP (3%) subjects and four healthy individuals (10%). HSV DNA and VZV DNA were not detected in the subset of studied individuals.

Conclusions: This study challenges the previously reported high prevalence of herpesvirus DNA in subgingival samples from periodontitis patients and so questions whether they act as pathogens in such patients.

Key words: aggressive; herpes; pathogenesis; periodontitis; virus

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Growing evidence suggests that certain viruses may play a role in the pathogenesis of periodontitis. In particular, DNA from herpesviruses such as human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) has been detected in high percentages of subgingival plaque samples from periodontitis patients. In contrast, very low prevalence of such viruses has been detected in periodontally healthy individuals (Contreras & Slots 1996, Yapar et al. 2003). Furthermore, herpesviruses have been associated with severity and activity of periodontitis and with presence of periodontopathogenic bacteria (Michalowicz et al. 2000, Kamma & Slots 2003). A recent review suggested that viruses may directly induce immunosuppression and may have direct cytopathic effect on fibroblasts, keratinocytes and inflammatory cells (Slots 2005). However, large studies confirming the association between periodontitis and presence of subgingival viruses are still lacking. The aim of this study was to analyse the presence of herpesviruses in subgingival pockets and the presence of immunoglobulin G (IgG) against the same viruses in serum samples in a large prevalently Caucasian population of periodontitis and in healthy individuals.

Conflict of interest and source of funding statement

This work was undertaken at UCLH/UCL who received a proportion of funding from the Department of Health's NIHR Biomedical Research Centres funding scheme. We declare we have no conflict of interest.

Materials and Methods

Study population

The study had a case–control design, with a total of 140 participants. A 100 cases were selected among patients referred to the Eastman Dental Hospital, University College London by general dental practitioners. Forty unmatched healthy controls were recruited among patients referred to other Departments of the Hospital. All of the patients gave written informed consent and the study had been reviewed and approved by the Joint UCL/UCLH Committees on the Ethics of Human Research.

All participants in the study had no signs or symptoms of systemic infection or disease as assessed by the examining clinician and had not taken any systemic antimicrobials in the last 3 months.

Inclusion criteria for periodontitis patients were

- Presence of at least three $\geqslant 5$ mm probing pocket depths (PPD) and clinical attachment loss (CAL) in at least three different quadrants, with radiographic evidence of bone loss.

Inclusion criteria for control subjects were

- Absence of any $\geqslant 5$ mm PPD and lifetime cumulative attachment levels (LCAL) (except or distal surfaces of second molars in case of impacted third molars).
- History of periodontitis and periodontal treatment.

Exclusion criteria included (i) known systemic diseases (cardiovascular, respiratory, renal, malignancy, etc.), (ii) history and/or presence of any other infections, (iii) systemic antibiotic treatment in the preceding 3 months, (iv) long-term treatment with any medication suspected to affect the periodontium (e.g. non-steroidal anti-inflammatory drugs), (v) pregnant or lactating females and (vi) <20 teeth present.

Clinical examination

One clinician (L. N.) assessed all subjects and assigned a diagnosis. A comprehensive clinical examination was performed by two single calibrated examiners (U. D. and T. R.), who collected full-mouth measures of PPD, recessions (REC) and LCAL were obtained at six points per tooth. Diagnosis of alveolar bone loss was confirmed by radiographic assessment from each patient. Patients were diagnosed as having localized aggressive periodontitis (LAgP), generalized aggressive periodontitis (GAgP) or chronic periodontitis (CP), according to the 1999 Consensus Classification (Armitage 1999).

Virus analysis

Samples of subgingival periodontal plaque were collected using a sterile curette from the four deepest pockets, one in each quadrant (Mombelli et al. 1991). Supragingival plaque was gently removed with a scaler, the site isolated from saliva and the curette inserted in the pocket. Samples were placed into 1 ml of reduced transport fluid (pooled sample) (Syed & Loesche 1972). Specimens were stored in a -70°C freezer under code until tested. Five samples were harvested in duplicate and placed in 1 ml reduced transport fluid and in 1 ml viral transport medium (a balanced salt solution containing penicillin and fungizone to prevent bacterial or fungal overgrowth), to assess whether differences in viral DNA detection due to different transport fluids could be detected. However, no differences in detection between the two media were observed. Total nucleic acid was extracted from the specimens using Boom's method by Nuclisens® easyMAG® platform (BioMérieux, Basingstoke, UK), an automated extraction system with magnetic micro-particle processing. Briefly, 110 μl of sample was added to 2 ml lysis buffer before extraction according to the manufacturers's instructions. Total nucleic acid was eluted in a 25 μl volume. Real-time TaqMan® PCR (Applied Biosystems, Warrington, UK) was carried out for the detection and quantitation of virus. An in-house assay specific for the highly conserved region of glycoprotein B was used for detection of CMV DNA (Mattes et al. 2005). Serial dilutions of cloned CMV glycoprotein B plasmid (from 200 to 2×10^6 copies/ml) were included for accurate quantification of the CMV viral load. The lower limit of detection for the assay was 100 copies/ml of sample. A previously described real-time PCR (Jabs et al. 2001) was used for detection and quantification of EBV DNA. Detection is achieved by the amplification of the BAMHI-K rightward open reading frame encoding EBNA-1. Serial dilutions of known concentration EBV DNA (derived from EBV transformed Raji cells) were included for accurate quantification of viral load. The lower limit of detection of the assay was 100 copies/ml of sample.

On a subset of 20 individuals with AgP (14 GAgP and 6 LAgP) an exploratory analysis was performed to detect herpes simplex (HSV) and varicella zoster virus (VZV) DNA in subgingival plaque samples, as well as HSV IgG and VZV IgG in serum samples. Qualitative HSV detection was achieved using an in-house HSV type-specific multiplex assay for the gB region of HSV. This assay uses two type-specific probes, each labelled with a different marker dye and allows simultaneous detection and typing of the HSV in the same reaction. Qualitative VZV detection was achieved by the use of the Artus VZV TM PCR kit (Artus, Qiagen, Hilden, Germany), a ready-to-use kit that contains all reagents and enzymes for the specific-real time amplification of the VZV genome.

Positive and negative controls were included on each run. All positive samples were repeated to confirm positivity. Results were analysed and viral loads calculated using the Sequence Detection System software on the ABI 7000/7500 platform (Applied Biosystems). Stringent laboratory practices were taken at every step to prevent false positive reactions through contamination. A minimum of two water samples were co-extracted on the Nuclisens® easyMAG® on each run with the specimens. The water samples were included on the Real-time PCR assays to detect contamination resulting from the extraction stage. Clean laboratory coats and disposable gloves were worn when handling samples and setting up PCR reactions. No template controls were included on every real-time PCR to detect amplicon contamination.

Serum IgG assays

Blood samples were collected via a single venipuncture. Serum was separated from the blood samples within 1 h of collection and stored in a -70°C freezer until tested. CMV, EBV and VZV serostatus was determined using an indirect immunoassay for IgG against inactivated human CMV antigen, EBV viral capsid antigen (VCA) and inactivated VZV antigen on the Liason analyzer (DiaSorin, Bracknell, UK), an automated enzyme immunoassay system. HSV serostatus was determined by the use of ETI-HSVK-G 1/2 manual enzyme immunoassay (DiaSorin) according to the manufacturers's instructions.

Statistical analysis

The SPSS 12.0 package was used for statistical analysis and the α value was set at 0.05. Continuous, normally distributed variables are reported as means $\pm$ standard deviations (SD). Comparisons of categorical and continuous data between groups were analysed with Chi-square test or Fisher's exact test and ANOVA, respectively. The outcome variable was the presence of individuals' viruses (HCMV, EBV) and IgG (HCMV IgG, EBV IgG).

Table 1. Demographic and clinical characteristics of the patients included in the study

	LAgP ($n = 16$)	GAgP ($n = 64$)	CP ($n = 20$)	Total periodontitis ($n = 100$)	Controls ($n = 40$)	Comparison periodontitis *versus* healthy
Age	27.7 ± 7.3	33.5 ± 5.1	43.4 ± 11.4	34.4 ± 8.6	50.3 ± 13.4	$p < 0.001$ ANOVA
Gender						
Male	6 (37.5%)	21 (32.8%)	10 (50.0%)	37 (37.0%)	20 (50.0%)	$p = 0.184$ Fisher's exact test
Female	10 (62.5%)	43 (67.2%)	10 (50.0%)	63 (63.0%)	20 (50.0%)	
Ethnicity						
Caucasian	6 (37.5%)	32 (50.0%)	11 (55.0%)	49 (49.0%)	39 (97.5%)	$p < 0.001$ Pearson's χ^2 test
Black	5 (31.3%)	24 (37.5%)	3 (15.0%)	32 (32.0%)	1 (2.5%)	
Asian	2 (12.5%)	4 (6.3%)	4 (20.0%)	10 (10.0%)	0	
Other	3 (18.8%)	4 (6.3%)	2 (10.0%)	9 (9.0%)	0	
Smoking						
No smokers	11 (68.8%)	30 (46.9%)	9 (45.0%)	50 (50.0%)	28 (70.0%)	$p = 0.034$ Pearson's χ^2
Former smokers	4 (25.0%)	20 (31.3%)	6 (30.0%)	30 (30.0%)	4 (7.5%)	
Current smokers	1 (6.2%)	14 (21.9%)	5 (25.0%)	20 (20.0%)	8 (22.5%)	
Periodontal clinical data						
No. of teeth	29.3 ± 1.0	28.4 ± 2.6	26.5 ± 3.8	28.1 ± 3.0	27.4 ± 2.6	$p = 0.221$ ANOVA
Average PPD	3.5 ± 0.6	4.0 ± 0.9	3.8 ± 0.9	3.9 ± 0.9	2.7 ± 1.0	$p < 0.001$ ANOVA
Average CAL	3.8 ± 0.5	4.2 ± 1.0	4.2 ± 1.0	4.1 ± 0.9	2.7 ± 1.1	$p < 0.001$ ANOVA

PPD, probing pocket depths; CAL, clinical attachment loss.

Results

The demographic and clinical characteristics of the study sample are presented in Table 1. Twenty individuals were diagnosed with CP, while 16 were classified as having LAgP, and 64 as GAgP. Forty subjects were classified as periodontally healthy. Cases and controls were unbalanced for age, ethnicity and smoking. In particular, controls were on average older and included higher percentages of Caucasians and non-smokers. HSV and VZV were not detected in any of the subset of 20 studied samples (data not presented in tables). Table 2 reports detection rates for EBV and HCMV in plaque samples and for anti-EBV and anti-HCMV IgG in serum samples in subjects divided by periodontal diagnosis. HCMV DNA was not detected in any individuals. EBV DNA was detected in 10 subjects, four of which were LAgP (25.0%), two were GAgP cases (3.1%) and four were healthy (10.3%). Overall, respectively, 5/86 (6%) Caucasians, 2/10 (20%) Asians, 3/33 (9%) Black subjects were positive for EBV. Eighty-five per cent and 95% of cases and controls, respectively, tested positive for anti-EBV IgG, while 59% and 70% of cases and controls were positive for anti-HCMV IgG. Four of the six AgP EBV-positive subjects had < 1200 copies/ml, while only two cases (one Black Caribbean and one Asian subject) had more elevated counts (17,477 and 166,000 copies/ml, respectively). The number of EBV DNA copies in positive controls varied from 1,654 to 320,895 copies/ml. LAgP cases had significantly more EBV detected than GAgP and CP cases ($p = 0.013$ and 0.031, respectively, at Fisher's exact test). However, no statistically significant differences between cases and controls were detected.

Table 2. Detection rate of viral DNA in subgingival plaque samples and of IgG in serum samples

	LAgP ($n = 16$)	GAgP ($n = 64$)	CP ($n = 18$)	Total periodontitis ($n = 100$)	Controls ($n = 40$)	Comparisons periodontitis *versus* healthy (Fisher's exact test)
PCR						
EBV	4 (25.0%)	2 (3.1%)	0	6 (6.0%)	4 (10.3%)	$p = 0.467$
HCMV	0	0	0	0	0	–
IgG-serology						
EBV	11 (84.6%)	54 (87.1%)	16 (94.1%)	81 (85.3%)	37 (94.9%)	$p = 0.343$
HCMV	12 (92.3%)	39 (62.9%)	13 (81.3%)	64 (70.3%)	23 (59.0%)	$p = 0.227$

EBV detection:
LAgP *versus* GAgP $p = 0.013$ Fisher's exact test.
LAgP *versus* CP $p = 0.031$ Fisher's exact test.
LAgP *versus* controls $p = 0.212$ Fisher's exact test.
CP *versus* controls $p = 0.289$ Fisher's exact test.
GAgP *versus* controls $p = 0.196$ Fisher's exact test.
LAgP, localized aggressive periodontitis; GAgP, generalized aggressive periodontitis; CP, chronic periodontitis; PCR, polymerase chain reaction; EBV, Epstein–Barr virus; HCMV, human cytomegalovirus.

Discussion

Periodontitis is an inflammatory disease determined by the presence of microorganisms in the subgingival crevice, able to induce an inflammatory response, eventually leading to resorption of alveolar bone and periodontal ligament. Among infective agents able to trigger periodontitis, some Gram-negative bacteria have been isolated (AAP 1996), and some others are suspected. In the last 10 years, certain viruses have been proposed as possible periodontopathogens (Contreras & Slots 1996). The main evidence for this comes from epidemiological studies, which identified the presence of herpesviruses in subgingival pockets of patients with periodontitis. Herpesviruses are DNA double-stranded enveloped viruses, including among others HSV 1 and 2, VZV, EBV and HCMV. Studies comparing the detection of herpesvirus DNA in periodontitis and healthy subjects reported high

prevalence of HCMV and EBV in patients with chronic periodontitis (Contreras & Slots 1996, Saygun et al. 2002) and aggressive periodontitis (Yapar et al. 2003, Saygun et al. 2004, Botero et al. 2007). Other studies have proposed an association between detection of these viruses and recognized periodontopathogenic bacteria in periodontitis patients (Michalowicz et al. 2000), and between detection of viruses and periodontal disease activity (Kamma & Slots 2003). The ability of these herpesviruses to induce immunosuppression and a possible direct cytopathic effect on fibroblasts, keratinocytes, inflammatory cells have been proposed as possible mechanisms of periodontal pathogenesis (Slots 2005).

We analysed the presence of HCMV and EBV DNA subgingivally in a sample of 140 subjects with periodontal phenotypes ranging from aggressive periodontitis, to moderate chronic periodontitis and to healthy periodontium. Not a single case tested positive for HCMV, while only 6% of periodontitis patients and 10% of controls harboured EBV. This is the first study to report an analysis of VZV in plaque samples in periodontitis patients. However, all 20 tested subjects tested negative for VZV presence. These results are in clear conflict with previous literature results (Kamma & Slots 2003, Yapar et al. 2003, Botero et al. 2007). Reasons for such differences may involve study methods or differences in the analysed populations. The sampling method we used is standardized and has shown detection of periodontopathogenic bacteria comparable with literature data in a similar AgP population (Nibali et al. 2007). PCR analysis was the chosen method for detection of viral DNA, while nested PCR had been used in some previous studies. The latter has been shown to be more sensitive, but may carry the risk for detection of false positives (Botero et al. 2008). However, studies with PCR and comparable detection limits have yielded results significantly different from our study (Yapar et al. 2003). On the other hand, the discrepancies between our results and previous reports may be due to different studied populations. In particular, most studies have been performed in populations from South America, China, Turkey or in African Americans (Michalowicz et al. 2000, Yapar et al. 2003, Botero et al. 2007, Wu et al. 2007), with lack of studies on prevalently Caucasian populations, as the one described here. Rotola et al. (2008) found very low prevalence of HCMV in gingival biopsies in Caucasians and suggested that discrepancies with studies from non European Caucasian populations may be due to different prevalence of infections in different ethnic groups. The same authors also highlighted that low amounts of HHV-7 and EBV virus were harboured in biopsy specimens, and these were only detected by the very sensitive nested PCR technique (Rotola et al. 2008). Furthermore, the study we report here investigated a sample size far larger than most other studies. One hundred and forty individuals (100 of which were diagnosed with periodontitis) were included in our study, while other studied sample sizes range from 11 (Ting et al. 2000) to 143 periodontitis patients (Wu et al. 2007). It is also interesting to notice the higher prevalence of EBV in LAgP subjects (25%). Although this needs to be taken cautiously because of the small sample of LAgP ($n = 16$), this is somehow more consistent with literature data, reporting higher prevalence of viruses in LAgP cases (Ting et al. 2000). This latter study included LAgP cases that were younger than in our study and were prevalently African Americans. Out of 11 individuals, eight tested positive for HCMV DNA and seven were positive for EBV DNA. It is impossible to ascertain whether periodontal disease was active in the studied individuals presented here, or whether viruses could have been present at an earlier stage of periodontal destruction. However, all subjects had not had any periodontal treatment for at least 6 months, and had high levels of average PPD and bleeding on probing, a surrogate measure of disease activity. The discrepancy between the high prevalence of serum IgG positive for EBV and HCMV and the low detection in plaque samples may also be partly due to the characteristic latency of herpesviruses.

Within the limitations of an ethnically mixed population and a relatively small sample size, this study shows very low prevalence of subgingival herpesviruses in periodontal lesions. Only LAgP cases had a moderate prevalence of EBV, however, not significantly different from the control populations. Based on this study, we conclude that a high prevalence of subgingival viruses in periodontitis cases is not universal, but may depend on the studied population and to some extent on the methods used. Therefore, this study questions the supposed pathogenic and clinical relevance of virus infection in periodontitis.

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Clinical Relevance

Scientific rationale for study: Viruses have been recently implicated in the pathogenesis of periodontitis
Principal findings: Only a small subset of subjects were found to harbour viruses in subgingival plaque samples. No differences between periodontitis patients and controls were detected.
Practical implications: Viruses may not have a high prevalence in subgingival plaque samples in all populations.