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Quantitative analysis of association between herpesviruses and bacterial pathogens in periodontitis

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Background and Objective: The development of human periodontitis may depend upon cooperative interactions among herpesviruses, specific pathogenic bacteria and tissue-destructive inflammatory mediators. This study sought to identify associations among human cytomegalovirus, Epstein–Barr virus and six putative periodontopathic bacteria in periodontitis lesions.

Material and Methods: Fifteen periodontitis patients (nine with aggressive periodontitis and six with chronic periodontitis) and 15 periodontally normal subjects were included in the study. In each study subject, a microbiological sample was collected, using a curette, from the deepest periodontal probing depth of the dentition. A real-time TaqMan® polymerase chain reaction assay was employed to determine the subgingival counts of human cytomegalovirus, Epstein–Barr virus, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Campylobacter rectus*. Statistical analysis was performed using the Student's *t*-test, the Pearson correlation coefficient test and the single variable logistic regression test for odds ratio-based risk calculation.

Results: Human cytomegalovirus was detected in eight periodontitis lesions and in one normal periodontal site, Epstein–Barr virus was detected in nine periodontitis lesions and in two normal periodontal sites, and the study bacteria were detected in 6–15 periodontitis lesions and in 1–11 normal periodontal sites. Correlations were found between counts of human cytomegalovirus and Epstein–Barr virus, between counts of human cytomegalovirus and *P. gingivalis*, *T. forsythia* and *C. rectus*, and between counts of Epstein–Barr virus and *P. gingivalis* and *T. forsythia*. Human cytomegalovirus and Epstein–Barr virus counts were also positively associated with the level of periodontal attachment loss, probing pocket depth and gingival bleeding on probing.

Conclusion: This study confirmed that periodontal human cytomegalovirus and Epstein–Barr virus are associated with major periodontopathic bacteria and with the severity of periodontal disease. The finding of abundant herpesviruses in periodontitis lesions redefines the pathogenic paradigm of the disease. Understanding the interplay between herpesviruses and specific bacterial species in the pathogenesis of periodontitis may form the basis for new approaches to preventing, reducing or delaying tissue breakdown from periodontal infections.

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Periodontitis has traditionally been described as a bacterial infection caused by specific pathogenic species (1). Healthy gingival sites harbor a scant microbiota of mainly facultative, fermentative, gram-positive bacteria, whereas periodontitis lesions contain predominantly anaerobic, proteolytic, gram-negative species (2). The population shift in the periodontal microbiota with disease development is widely recognized but has never been fully explained. Research has identified potential pathogenic properties of periodontal bacteria (3) and environmental and host modifiers of periodontal disease (4). However, it is not known if bacteria cause periodontitis solely on their own, or if they play a part in the disease process as a microbiological consequence of some other pathophysiological events.

A major disturbance in the periodontal ecosystem must take place to cause the shift from predominantly a gram-positive facultative to a gram-negative anaerobic microbiota. The gram-positive species induce the development of gingivitis with a nutritionally rich serum exudate (5) and mediate the oral attachment of proteolytic gram-negative bacteria (6,7). Microbial factors, such as synergistic and antagonistic bacterial interactions, as well as host-specific factors, including innate and adaptive immune functions, also influence the composition of the periodontal microbiota. However, it is not clear why less-fastidious viridans streptococci and facultative actinomyces species would be outcompeted in inflamed periodontal sites. After all, various gram-positive facultative species proliferate successfully in inflamed endodontic lesions (8). Also, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* in localized aggressive (juvenile) periodontitis overwhelms the prediseased gram-positive microbiota at a time of minimal gingivitis and serum exudate (9). Moreover, periodontopathic gram-negative bacteria must overcome the inhibitory action of bacteriocins from coresident gram-positive species (10) and high levels of specific serum antibodies (11,12). Oral streptococci and actinomyces do not appear to face similar challenges from gram-negative plaque bacteria or from the host. It seems that the profound microbial changes during periodontitis development may not be accounted for solely by the appearance of unique microbial nutritional factors or metabolic activities within the subgingival biofilm.

Various clinical characteristics of periodontitis are also difficult to explain solely on the basis of a bacterial cause of the disease. In most patients, periodontitis affects a limited number of teeth despite an omnipresence of periodontopathic bacteria in saliva (13). Also, many periodontitis lesions are self-limiting with short-duration morbidity and do not proceed to tooth loss. The infectious basis for periodontitis disease initiation, exacerbation and stability remains obscure. Furthermore, as clearly evidenced in localized aggressive periodontitis, periodontal tissue destruction tends to occur in a bilateral symmetrical pattern in a dentition, and interproximal sites may show alveolar bone loss close to the apex at one tooth but virtually no bone breakdown at the neighboring teeth. The conventional explanation is that periodontitis-prone teeth exhibit anatomies predisposing to enhanced plaque accumulation. However, evidence linking *supra*gingival plaque accumulation with periodontitis disease severity is weak and inconsistent (14). Also, the plaque amount on the surfaces of neighboring teeth in the same interproximal space is not likely to vary sufficiently to explain vast different tissue responses. As periodontal pockets of all morphologic types amass high microbial densities, variation in the mere anatomy of *sub*gingival sites is also unlikely to constitute a major determinant of periodontitis severity.

It may be that different hypotheses of etiology fit separate parts of the periodontal disease process. There is little doubt, as inferred from experimental gingivitis studies, that the common type of chronic gingivitis can develop solely as a result of bacterial action. However, given the lack of direct evidence for a bacterial etiology of periodontitis and the many puzzling clinical realities of the disease, we suggest that a purely bacterial cause of periodontitis has been overemphasized. The development of periodontitis may depend on a successive occurrence of various cooperating infectious events (15). The finding of abundant herpesviruses in severe periodontitis lesions may provide important insights into the causation of the disease (16,17). A concurrent herpesviral–bacterial infection is likely to exert more periodontopathogenecity than single infections by either of the two types of infectious agents (18). The observed positive association between periodontal herpesviruses and pathogenic bacteria may indicate a potential of viral infections to predispose to bacterial carriage or to increase the bacterial load (18).

To confirm and extend previous findings of herpesviruses in periodontitis, this study employed a real-time polymerase chain reaction (PCR) assay to determine possible correlations among periodontal pocket counts of human cytomegalovirus, Epstein–Barr virus and various putative periodontopathic bacteria. Previous studies on herpesviral–bacterial interactions in periodontal disease utilized qualitative PCR methodologies, which may have underestimated the strength of association between periodontal infectious agents.

Material and methods

Subjects

The study included nine patients (aged 19–33 years) with aggressive periodontitis, six patients (aged 32–49 years) with chronic periodontitis and 15 periodontally normal subjects (aged 31–42 years). The 30 study participants were scheduled for periodontal examination at the Department of Periodontology, Gülhane Military Medical Academy (Ankara, Turkey). All patients were systemically healthy and had not received periodontal treatment or antibiotics for at least 6 mo prior to the start of the study. The Institutional Internal Review and Ethics Board at the Gülhane Military Medical Academy, Sciences of Dentistry, approved the study. Written informed consent was obtained from each study subject

after all procedures had been fully explained.

Clinical procedures

Clinical periodontal evaluation included determination of plaque index (19), gingival index (20), the percentage of sites that bled on probing, probing pocket depth and probing attachment loss. Probings were carried out using a Williams probe calibrated in millimeters and were assessed at four sites per tooth: mesiofacial; midfacial; distofacial; and midlingual. In each study patient, a subgingival sample was obtained from the deepest pocket of the dentition. Prior to sampling, the sample site was gently cleaned of supragingival plaque and saliva using sterile cotton pellets, isolated with cotton rolls and air dried. A sterile periodontal curette was gently inserted until the bottom of the test periodontal pocket and subgingival material was removed with a single stroke. The subgingival samples were suspended in 500 μL of 10 mM Tris–HCl containing 1 mM EDTA (pH 8) (TE buffer) and homogenized by vigorous mixing on a vortex.

Nucleic acid extraction

DNA was extracted from the clinical sample material using an alkali phenol–chloroform–isoamyl alcohol procedure (21). Briefly, 100 μL of specimen was placed in 10 μL of protease solution (65 mg/mL) (Sigma-Aldrich Corp., St Louis, MO, USA) and 250 μL of potassium buffer for 60 min at 42°C. Following centrifugation at 10,000 *g* for 10 min at 12°C, DNA was extracted from the supernatant using a mixture of 250 μL of alkali phenol and 250 μL of chloroform–isoamyl alcohol (25 : 24 : 1), and then precipitated using 500 μL of isopropyl alcohol. DNA was washed in 75% ethyl alcohol at 10,000 *g* for 5 min at 4°C, air dried at 37°C, and dissolved in 100 μL of distilled water.

Real-time TaqMan® assay

The TaqMan® fluorogenic real-time PCR detection system was used to determine infectious agent counts (22). The TaqMan system uses species-specific primers and probes that are dually labeled with a fluorescent reporter and a quencher dye. The *Taq* polymerase cleaves the TaqMan probe during the PCR amplification process, separating the reporter from the quencher dye, which increases the intensity of the reporter fluorescence proportionally to the starting copy counts of the target DNA. Table 1 lists the nucleotide

Table 1. Polymerase chain reaction primers and TaqMan probes for detecting herpesviruses and bacteria

Infectious agents (GenBank accession number)		Amplicon size in bp (reference)
Human cytomegalovirus (AY446894.1)		84 (24)
Forward:	5′-TGAGCCCGGCGGTGGT-3′	
Reverse:	5′-AGCTCACCGATCACAGACAC-3′,	
Probe:	5′-FAM-AGAGAAGCGCCACATACAGCGC-TAMRA-3′	
Epstein–Barr virus (V01555.2)		95 (24)
Forward:	5′-CCTGGTCATCCTTTGCCA-3′	
Reverse:	5′-TGCTTCGTTATAGCCGTAGT-3′,	
Probe:	5′-FAM-CAGTACGAGTGCCTGCGACCA-TAMRA-3′	
Porphyromonas gingivalis (AB261608.1)		194 (this study)
Forward:	5′-TGGGACTTGCTGCTCTTGCTATG-3′	
Reverse:	5′-GATGGCTTCCTGCTGTTCTCCA-3′	
Probe:	FAM-5′-CAAAGACAACGAGGCAGAACCCGTTA-TAMRA-3′	
Tannerella forsythia (DQ344918.1)		149 (this study)
Forward:	5′-GCGTATGTAACCTGCCCGCA-3′	
Reverse:	5′-CCGTTACCTCACCAACTACCTAATG-3′	
Probe:	FAM-5′-AGGGATAACCCGGCGAAAGTCGGA-TAMRA-3′	
Prevotella intermedia (AY689226.1)		105 (this study)
Forward:	5′-AGACGGCCTAATACCCGATGTTG-3′	
Reverse:	5′-TTACCCGCACCAACAAGCTAATCAG-3′	
Probe:	JOE-5′-TGGCATCTGACGTGGACCAAAGATTC-TAMRA-3′	
Aggregatibacter actinomycetemcomitans (AF359451.1)		288 (this study)
Forward:	5′-CGGTTACCGTTATGACCGTGTGA-3′	
Reverse:	5′-GCCCGGAATGCTTTGCTATATTTC-3′	
Probe:	FAM-5′-AGGCAAGACGGGAAGCTAACGCAAA-TAMRA-3′	
Fusobacterium nucleatum (EF089177.1)		175 (this study)
Forward:	5′-GCGGAACTACAAGTGTAGAGGTG-3′	
Reverse:	5′-GTTCGACCCCCAACACCTAGTA-3′	
Probe:	JOE-5′-AATGCCGATGGGGAAGCCAGCTTA-TAMRA-3′	
Campylobacter rectus (AF035193.1)		132 (this study)
Forward:	5′-CACCCGATAACCCTACTCCTCCTA-3′	
Reverse:	5′-GATCCGTTCCATCAGTACCCACTA-3′	
Probe:	FAM-5′-CCGGTACCGAATCCTGAGGAACCA-TAMRA-3′	

FAM, 6-carboxyfluorescein labeled reporter dye; JOE, 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein labeled reporter dye; TAMRA, 6-carboxytetramethyl-rhodamine labeled quencher dye.

sequence of the PCR primers and probes, which were designed using the OLIGOWARE 1.0 software program (23). Study infectious agents included human cytomegalovirus, Epstein–Barr virus, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *A. actinomycetemcomitans*, *Fusobacterium nucleatum* and *Campylobacter rectus*. PCR amplification was performed as individual assays for each infectious agent studied. Amplification, data acquisition and all analyses were carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Standard procedures for the operation of the model 7700 system were followed, including the use of all default program settings. For each infectious agent tested, the TaqMan PCR assay was performed with a final volume of 25 μL of reaction mixture, containing 5 μL of extracted clinical sample, 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 5 pmol of primers and 3 or 4 pmol of TaqMan probe. The PCR cycling program included 2 min at 50°C to eliminate carryover contamination, 10 min at 95°C to activate the hot-start *Taq* DNA polymerase (AmpliTaq Gold® DNA Polymerase; Applied Biosystems) and then 40 cycles, with each cycle consisting of two steps at 60°C for 1 min and one step at 95°C for 15 s. Herpesviral and bacterial copy numbers were multiplied by 100 to adjust for sample dilution in the PCR assay. PCR quantification standards included plasmids containing viral and bacterial amplicons, which were cloned using the TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). No cross-reactivity was observed among human cytomegalovirus, Epstein–Barr virus, herpes simplex virus types 1 and 2, human herpesvirus 6 and human herpesvirus 8 (data not shown). The test bacteria also showed no cross-reactivity with even closely related species, such as *Prevotella nigrescens*. Also, a BLAST search (National Center for Biotechnology Information, Bethesda, MD, USA) to check the specificity of the primers and probes used showed no genomic cross-reactivity with other mammalian viruses, bacteria or cells. The dynamic range of quantification of the TaqMan PCR assay was determined by serial dilution of the plasmid-generated standards in the range of 10^9–10^1 copies per mL.

Statistical analysis

Statistical evaluation was performed using the SPSS 10.0 statistical package (SPSS Inc., Chicago, IL, USA). Descriptive data were reported in frequency and percentage for categorical variables, and in mean and standard deviation for scale variables. Counts of the viruses and bacteria were normalized by conversion to logarithmic values. The Student's *t*-test was used for comparisons between subject groups. Pearson correlation coefficient analysis was used in correlation calculations (25). Single variable logistic regression analysis was used for odds ratio-based risk calculation. The presence of periodontitis comprised the dependent variable, and logarithmic-transformed data of the infectious agents constituted the independent variables. Probability (p) values equal to or less than 0.05 were considered statistically significant.

Results

Whole mouth examination of patients with periodontitis and of periodontally normal subjects revealed averages, respectively, of 3.8 and 1.7 mm in periodontal pocket depth, 4.0 and 1.7 mm in clinical attachment loss, 1.7 and 0.8 in plaque index, 1.6 and 0.7 in gingival index, and 61.4 and 8.2% of sites bleeding on probing. Microbiological sample sites of periodontitis patients and of periodontally normal subjects showed averages, respectively, of 4.3 and 1.9 mm in periodontal pocket depth, 4.8 and 1.9 mm in clinical attachment loss, 1.7 and 1.0 in plaque index, 1.9 and 0.7 in gingival index, and 78.3 and 11.7% of sites bleeding on probing. All clinical differences between the periodontitis patients and the periodontally normal subjects were statistically significant ($p < 0.001$).

Human cytomegalovirus was detected in eight periodontitis lesions and in one normal periodontal site, and Epstein–Barr virus was detected in nine periodontitis lesions and in two normal periodontal sites (Table 2). PCR-positive periodontitis lesions showed median human cytomegalovirus counts of 6.35×10^4 and median Epstein–Barr virus counts of 7.1×10^5 (Table 2). PCR-positive normal periodontal sites yielded $\approx$30-fold lower counts of the two viruses. *P. gingivalis* and *T. forsythia* were the most frequently detected bacteria in periodontitis lesions (Table 2).

Table 3 describes the relationship between infectious agents and risk for periodontitis, based on odds ratio calculations. Human cytomegalovirus, Epstein–Barr virus and all test bacteria, with the exception of *F. nucleatum*, increased the risk for periodontitis by magnitudes of 1.4–2.6, and the increases were statistically significant. *F. nucleatum* was detected at a similar frequency in periodontitis sites and in normal periodontal sites and showed a nonsignificant odds ratio of 1.2.

Several statistical correlations were detected among herpesviral counts, bacterial counts and clinical variables (Table 4). Positive correlations were found between human cytomegalovirus and *P. gingivalis*, *T. forsythia* and *C. rectus*. Epstein–Barr virus correlated with *P. gingivalis* and *T. forsythia*. Clinically, human cytomegalovirus and Epstein–Barr virus correlated with the level of gingival inflammation and periodontitis disease severity, as assessed by increased pocket depth and probing attachment loss. Epstein–Barr virus correlated negatively with patient age. *P. gingivalis*, *T. forsythia* and *C. rectus* were the only test bacteria that showed a significant correlation with both periodontal pocket depth and attachment loss, as analyzed by either whole mouth data or sample-site findings.

Discussion

The difficulty in determining critical pathogenic determinants of periodontitis stems from complexities in identifying the full spectrum of

Table 2. Occurrence of subgingival herpesviruses and bacterial species in periodontitis lesions and normal periodontal sites

	Periodontal lesions ($n = 15$)				Normal periodontal sites ($n = 15$)			
Infectious agents	No. (%) of PCR-positive sites	Median counts in PCR-positive sites	Range of counts in PCR-positive sites	Mean ± standard deviation (log)[a]	No. (%) of PCR-positive sites	Median counts in PCR-positive sites	Range of counts in PCR-positive sites	Mean ± standard deviation (log)[a]
Cytomegalovirus	8 (53%)	6.35×10^4	2.1×10^3–4.6×10^5	2.5 ± 2.5	1 (7%)	2.1×10^3	NA	0.2 ± 0.9
Epstein–Barr virus	9 (60%)	7.1×10^5	2.1×10^3–8.3×10^8	3.7 ± 3.5	2 (13%)	1.72×10^4	2.4×10^3–3.2×10^4	0.5 ± 1.4
Porphyromonas gingivalis	13 (87%)	8×10^6	5×10^3–1×10^{10}	6.5 ± 3.1	2 (13%)	1.5605×10^6	2.1×10^4–3.1×10^6	0.7 ± 2.0
Tannerella forsythia	15 (100%)	8×10^7	3.2×10^3–8×10^8	7.0 ± 1.8	3 (20%)	2.1×10^3	2.1×10^3–3.4×10^3	0.7 ± 1.4
Prevotella intermedia	7 (47%)	8×10^6	6×10^4–3×10^9	3.5 ± 4.0	2 (13%)	1.365×10^4	6.3×10^3–2.1×10^4	0.5 ± 1.4
Aggregatibacter actinomycetemcomitans	6 (40%)	2.63×10^6	5×10^4–2.1×10^8	2.4 ± 3.2	1 (7%)	2.1×10^4	NA	0.3 ± 1.1
Fusobacterium nucleatum	11 (73%)	3×10^8	4×10^5–2×10^9	5.8 ± 3.8	11 (73%)	6.1×10^4	5×10^3–3.1×10^7	4.0 ± 2.7
Campylobacter rectus	12 (80%)	1.73×10^6	1.3×10^3–3.2×10^8	4.8 ± 2.8	2 (13%)	1.865×10^4	1.3×10^3–3.6×10^4	0.5 ± 1.4

[a]All mean differences of infectious agents between periodontitis lesions and normal periodontal sites were statistically significant ($p = 0.03$–0.001), except for *Fusobacterium nucleatum* ($p = 0.13$).
NA, not applicable; PCR, polymerase chain reaction.

pathogenic agents and in distinguishing between destructive and protective features of the gingival inflammatory response. Using quantitative detection methods, this study found a parallel increase of *P. gingivalis* and *T. forsythia* subgingival counts with gingival inflammation and periodontal attachment loss. These findings are consistent with a periodontopathic role of those bacteria. However, data merely showing an association between changes in bacterial load and disease severity do not prove causality. It may be that a bacterial shift basically constitutes a secondary phenomenon to other key pathophysiological events. In fact, as demonstrated in this and other studies (18,26,27), the periodontal presence of human cytomegalovirus or Epstein–Barr virus was statistically associated with an elevated occurrence of periodontal pathogenic bacteria, especially *P. gingivalis* and *T. forsythia*, the two study species with the highest suspected pathogenic potential (3). Of interest, correlations with both human cytomegalovirus and Epstein–Barr virus were only found for *P. gingivalis* and *T. forsythia*. The obtained data strengthen the hypothesis of a causal relationship between herpesviral–bacterial combined infection and destructive periodontal disease and point to the potential of herpesviruses to unsettle the delicate balance between host immunity and specific bacteria.

It seems unlikely that the observed correlation between herpesviral counts and periodontal disease severity was solely caused by a passive and etiologically irrelevant influx of virus-infected inflammatory cells. In patients with aggressive periodontitis, Kamma *et al.* (28) found herpesviral presence to be related to disease-active periodontitis, but not to gingival bleeding. Also, Ting *et al.* (29) demonstrated a high occurrence of human cytomegalovirus and Epstein–Barr virus in early aggressive (juvenile) periodontitis lesions that showed little or no clinical inflammation. The negative correlation between Epstein–Barr virus and patient age shown here also points to viral involvement in the more aggressive type of periodontitis found in younger patients. The inclusion in this study of

Table 3. Subgingival infectious agents and risk of periodontitis[a]

Infectious agents	Odds ratio	95% CI	*p*-value
Cytomegalovirus	2.0	1.1–3.6	0.02
Epstein–Barr virus	1.6	1.1–2.4	0.01
Porphyromonas gingivalis	1.8	1.3–2.5	0.001
Tannerella forsythia	2.6	1.4–4.8	0.003
Prevotella intermedia	1.4	1.0–2.0	0.03
Aggregatibacter actinomycetemcomitans	1.6	1.0–2.5	0.05
Fusobacterium nucleatum	1.2	0.9–1.5	0.13
Campylobacter rectus	2.0	1.3–3.1	0.002

[a]Single variable logistic regression analysis.
CI, confidence interval.

adults with chronic periodontitis may explain the observed positive, although weak, correlation between herpesviral counts and gingivitis. As most adults exhibit considerable antiherpesviral immunity, herpesviruses in chronic periodontitis lesions may induce periodontopathogenic mediators in levels sufficient to support gingivitis but not to cause major attachment loss.

The involvement of herpesviruses in disease-active periodontitis challenges several conventional concepts about

Table 4. Correlations among subgingival infectious agents and periodontal clinical variables[a]

Infectious agents	Significant ($p < 0.05$) positive correlations with infectious agents	Significant ($p < 0.05$) positive correlations with whole mouth clinical variables	Significant ($p < 0.05$) positive correlations with clinical variables at sample sites
Cytomegalovirus	Epstein–Barr virus***	Pocket depth**	Pocket depth*
	*P. gingivalis**	Attachment loss**	Attachment loss*
	*T. forsythia**	Gingival index*	Gingival index*
	*C. rectus**	Bleeding on probing*	Bleeding on probing*
Epstein–Barr virus	Cytomegalovirus***	Patient age (negative correlation)*	Pocket depth*
	*P. gingivalis**		Attachment loss*
	*T. forsythia**	Pocket depth**	Gingival index*
		Attachment loss**	Bleeding on probing*
		Plaque index*	
		Gingival index*	
		Bleeding on probing**	
Porphyromonas gingivalis	Cytomegalovirus*	Pocket depth***	Pocket depth*
	Epstein–Barr virus*	Attachment loss***	Attachment loss**
	*T. forsythia****	Plaque index***	Plaque index*
	*P. intermedia**	Gingival index**	Gingival index**
	*A. actinomycetemcomitans**	Bleeding on probing***	Bleeding on probing***
	*C. rectus****		
Tannerella forsythia	Cytomegalovirus*	Pocket depth***	Pocket depth***
	Epstein–Barr virus*	Attachment loss***	Attachment loss***
	*P. gingivalis****	Plaque index***	Plaque index***
	*P. intermedia**	Gingival index***	Gingival index***
	*A. actinomycetemcomitans**	Bleeding on probing***	Bleeding on probing***
	*F. nucleatum**		
	*C. rectus***		
Prevotella intermedia	*P. gingivalis**	Pocket depth*	Plaque index*
	*T. forsythia**	Attachment loss*	Gingival index*
	*F. nucleatum**	Plaque index*	Bleeding on probing*
	*C. rectus***	Gingival index*	
Aggregatibacter actinomycetemcomitans	*P. gingivalis**	Pocket depth*	Attachment loss*
	*T. forsythia**	Gingival index*	Plaque index*
	*C. rectus**	Bleeding on probing*	Gingival index*
Fusobacterium nucleatum	*T. forsythia**	Patient age*	Plaque index*
	*P. intermedia**	Plaque index*	Bleeding on probing*
Campylobacter rectus	Cytomegalovirus*	Pocket depth**	Pocket depth*
	*P. gingivalis****	Attachment loss***	Attachment loss*
	*T. forsythia***	Plaque index*	Plaque index*
	*P. intermedia***	Gingival index**	Gingival index**
	*A. actinomycetemcomitans**	Bleeding on probing*	Bleeding on probing ***

[a]Pearson correlation coefficient analysis.
*Rho < 0.60; **Rho 0.60–0.70; ***Rho > 0.70.
A. actinomycetemcomitans, *Aggregatibacter actinomycetemcomitans*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *T. forsythia*, *Tannerella forsythia*.

periodontal immunology. Both the innate and the adaptive immune system play a role in preventing herpesviral replication and dissemination within the host (16,17,30). Briefly, a herpesviral active infection initiates the activation of nuclear factor-kappa B and its translocation to the nucleus, promoting the expression of pro-inflammatory cytokines, chemokines and adhesion molecules in virally infected cells. Pro-inflammatory cytokines recruit macrophages and natural killer cells to the site of infection and activate the cellular expression of various effector functions. Cells of the innate immune system lyse virally infected cells and are a rich source of T helper type 1 pro-inflammatory antiviral cytokines, including interleukin-1, interleukin-6, interleukin-12, interleukin-18, tumor necrosis factor-α and interferons. The concerted action of innate immune responses activates the adaptive immune system, especially $CD8^+$ cytotoxic T lymphocytes, in the defense against herpesviral infections. However, the pathophysiology of herpesviral infections also includes viral immunoevasins that exploit diverse cellular processes to interfere with host antiviral functions (16,17).

In extracellular bacterial infections, the innate immune system makes use of phagocytic cells, the complement system and natural antibodies (31,32). Mammalian cells recognize bacteria by means of Toll-like receptors, which lead to cellular activation and subsequent release of antibacterial defensins and cytokines of mainly the T helper 2 type. Bacterial infections sculpt the adaptive immune system to produce antibacterial immunoglobulins through the activation of B lymphocytes/plasma cells. The major mechanism for controlling periodontopathic bacteria appears to center on specific antibodies and polymorphonuclear leukocytes (32).

It may be that periodontitis develops because of dysregulated immune responses to the herpesviral–bacterial combined infection. As antiherpesviral and antibacterial immunity are partly antagonistic to each other, the diseased periodontium may experience a changing dominance of either herpesviral or bacterial immune responses. For example, T helper type 1 pro-inflammatory cytokines can undermine T helper type 2-mediated immunity, and vice versa (16,33). Immunosuppression may impair cellular immune defenses, which increases the risk of herpesviral re-activation and a subsequent spike in cytotoxic T cells and pro-inflammatory cytokines (34). Pro-inflammatory cytokines occur at elevated levels in severe periodontitis lesions (35), where they are linked to collagen degradation and bone resorption (36). Furthermore, the increase in cytotoxic/suppressor T cells in severe periodontitis lesions, which possibly is due to infecting herpesviruses, may adversely affect mammalian cells involved in the antibacterial defense (37). Also, herpesviruses are capable of subverting complement (38), neutrophil (39) and macrophage (40) functions, which may lessen the host defense against bacterial infections. *P. gingivalis* and other exogenous-like species, which are mainly controlled by antibody-mediated host responses (11), may particularly benefit from a reduction in antibacterial immunity and outgrow coresident indigenous microorganisms. Conceivably, as a herpesviral active infection has the potential to impair antibacterial defenses, it may trigger a microbial shift towards a more virulent subgingival flora, leading to periodontal tissue breakdown. If so, the (re)establishment of effective antiviral immunity may constitute an important aspect of achieving a long-lasting state of remission of progressive periodontitis.

Notably, even though pro-inflammatory cytokines have the potential to initiate collagen degradation and alveolar bone resorption (36), the periodontal cytokine response may actually be beneficial overall by preventing the activation and widespread dissemination of virulent viruses (41). Similarly, although cytotoxic/suppressor T cells may impede the antibacterial cellular defense, they also confer a critical antiviral function. As the diseased periodontium is a major reservoir for human cytomegalovirus and Epstein–Barr virus (42), perhaps periodontitis can teleologically be viewed as the biological price paid by the host to control periodontal herpesviruses and avoid viral dissemination and serious systemic diseases.

In summary, we propose that unfavorable changes in environmental exposure or alterations in genes of the immune system may periodically suppress the periodontal host defense, which then may lead to the re-activation of resident herpesviruses and increases in pro-inflammatory mediators, followed by the overgrowth of pathogenic bacteria. A further understanding of the role of herpesviruses in periodontitis may be crucial for elucidating the pathophysiology of the disease and for identifying novel and more efficacious targets for disease prevention and long-term cure. Future management of periodontal disease may benefit from antiherpesviral immunotherapeutics: either prophylactic vaccines, which harness the immune system of healthy subjects to prevent infection by disease-causing viruses; or therapeutic vaccines, which stimulate the immune system into combating existing viruses and disease.

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