Diagnosis of Periodontal Disease at the Periodontal Tissue-Biofilm Interface: Biological Correlates

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Purpose: Periodontal disease has traditionally been classified based upon history and clinical presentation, but considerable heterogeneity within each diagnostic category persists suggesting the need for improved periodontal classifications that better reflect the biology of the periodontal tissue-biofilm interface (PBI). The purpose of this study was to use biological parameters that are all influencing the local expression of disease at the PBI in combination with the clinical determinants of the periodontal tissue-biofilm interface to better understand the inflammatory and microbial phenotypes that may underlie clinical disease.

Materials and Methods: Data from an epidemiologic study that included full-mouth clinical periodontal measurements from 6793 community-dwelling subjects were analyzed to identify heterogeneity in disease presentation to create periodontal disease classifications based upon two measures more directly related to the PBI, probing depths (PD) and bleeding on probing (BOP) scores. This analysis ignored attachment loss measurements and focused on defining clinical disease based upon these two clinical measurements to describe the periodontal tissue biofilm interface. Four distinct PBI disease classifications emerged [Healthy (PBI-H), Gingivitis (PBI-G), moderate periodontitis (PBI-MP) and severe periodontitis (PBI-SP)]. The biological profile of these four disease groups was explored using serum antibody IgG levels, the level of periodontal organisms present and the gingival crevicular fluid (GCF) inflammatory mediator response.

Results: Subjects with PBI-H had lower levels of serum antibody, as compared to all diseased subjects. The level of organism present was similar for the healthy, gingivitis and moderate periodontitis subjects. PBI-G and PBI-MP had similar levels of serum antibody and microbes, but the PBI-SP group had higher levels of serum IgG and periodontal pathogens as well as elevated GCF levels of $IL_{-1}B$, PGE_2, MCP_{-1} and IL_{-6}.

Conclusions: Severe periodontitis differs biologically from moderate periodontitis beyond severity in clinical presentation. PBI-SP is associated with an increase in microbial load, an enhanced antibody response and an increase in the innate inflammatory response.

Key words: periodontal disease, diagnostics, gingival crevicular fluid

INTRODUCTION

Periodontal disease definitions and classifications are periodically reviewed and refined at both the European and American Periodontal Consensus conferences. The final clustering of clinical signs into specific, discrete disease categories continues to evolve (Machtei et al, 1992; Beck, 1998; Baelum and Lopez, 2003; Preshaw et al, 2004). These disease classifications serve as important guidelines for clinicians to evaluate a patient to reach an accurate diagnosis, a process that usually serves to direct therapeutic options. Ideally, this diagnostic process results in creating non-ambiguous, distinct borders that distinguish between disease categories and that each diagnostic category has an identified treatment algorithm that has been optimized and
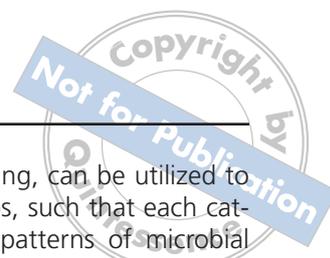
validated. Under ideal circumstances patients within each category are fairly homogeneous, such that diagnosis-based treatments can yield a predictable treatment outcome. Unfortunately, diagnostic categories for periodontal classification are based almost exclusively on a combination of history and clinical signs at presentation. Within a specific disease category there is considerable variability in the extent and severity of disease expression. Clinical signs, as evaluated individually or as combined into disease scores, have relatively poor sensitivity and specificity with regards to predicting disease outcomes in treated or untreated individuals.

Since clinical signs are a consequence of the interaction between the biofilm load and the host response we sought to approach this nosological task to develop diagnostic categories based upon what is happening at the interface. The periodontal pocket is bordered by the sulcular epithelium or pocket epithelium. Probing depth provides a reasonable approximation of the surface area especially when considered at six sites per tooth on all teeth. Thus, this measurement can define one attribute of the interface. When the epithelium is ulcerated or friable, bleeding on probing is the associated clinical sign. Thus these two clinical signs probing depth and bleeding on probing were used to define disease at the PBI. To determine the contribution of the biofilm load we determined the level of pathogens present within the periodontal pocket using DNA checkerboard methods. The host inflammatory response is represented by measuring the levels of biochemical mediators of inflammation within the gingival crevicular fluid. The patterns of these inflammatory mediators reveal the specific qualitative and quantitative aspects of the innate inflammatory response. Finally, the acquired immune response to the existing biofilm can be determined by using the serum antibody level to each pathogen. This purpose of this investigation was to begin to use these biological parameters that are all influencing the local expression of disease at the PBI in combination with the clinical determinants of the PBI to better understand the inflammatory and microbial phenotypes that may underlie clinical disease.

In this report we sought to reassess periodontal disease status using an existing datasets from a previous study. We sought to identify which components of clinical signs would enable us to segregate discrete clusters of individuals that could be considered as a new, clinically relevant diagnostic category. We then ascertained which exposures or clinical characteristics seemed to best describe the extent or severity of the condition within each diagnostic category. Important to this process was the finding that not all clinical signs were informative in terms of defining a disease cluster or explaining the extent of disease. Our findings indicate that distilling the data down to two clinical signs - prob-

ing depths and bleeding on probing, can be utilized to create disease classification groups, such that each category has significantly different patterns of microbial burden and host response that would have been indistinguishable using traditional disease classifications. Although a full explanation of these findings is beyond the scope of this limited report, we provide a synopsis of the key clinical and biological findings.

MATERIALS AND METHODS

Periodontal Assessments in Population-Based Study

Full-mouth periodontal examination data were gathered on 6793 individuals living in four US communities. This cross-sectional assessment of individuals also included detailed medical history data and has been described in detail in several earlier publications (Elter et al, 2004, 2005; Beck et al, 2001). Full-mouth periodontal examinations were performed on all teeth including third molars. Clinical assessments included Plaque Index (Loe and Silness, 1963) and the Gingival Index (Silness and Loe, 1964), which were collected by calibrated examiners (put in agreement levels). Probing depths, CEJ measurements and attachment levels (computed from PD and CEJ) were determined in mm at six sites per tooth, as was bleeding on probing (yes/no) at each site. The extent of bleeding on probing (BOP) was expressed as a percent of all sites.

Periodontal disease at the periodontal tissue-biofilm interface (PBI) was defined using probing depths and bleeding on probing. For these purposes attachment level measurements were not used as it reflects a historic assessment of disease progression and does not add information regarding the biology of the PBI beyond that which is captured by the probing depth measurement. Since all subjects in this population had some attachment loss and could be considered as having periodontitis we use the term PBI as a prefix to clarify that the clinical state is defined without consideration of attachment loss. Four levels of disease were defined based upon either low or high pocket depths and either low or high bleeding scores. PBI-Healthy (PBI-H) was defined as no probing depths of 4 or more mm and <10% BOP (972 Subjects). PBI-Gingivitis (PBI-G, 1026 Subjects) was defined as individuals with no probing depths of 4 or more mm and ≥10% BOP. Two separate periodontitis PBI groups were created based upon whole mouth PD and BOP scores. PBI-Moderate periodontitis (PBI-MP) (3916 subjects) was defined as one or more sites with PD≥4mm and BOP extent scores <50% and PBI-severe periodontitis (PBI-SP) (879 subjects) as subjects with one or more sites with PD≥4mm and BOP extent scores ≥50%.
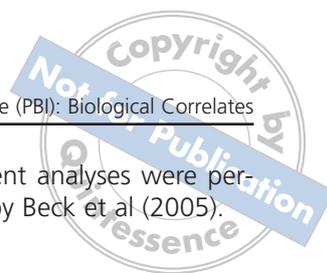
Gingival Crevicular Fluid Analyses

GCF was collected as described in detail previously (Champagne et al, 2003). Four GCF strips were eluted and analyzed separately (from the mesiolingual of each first molar) from each subject and pooled to provide a patient mean value for each analyte. For GCF analyses a random sample of subjects were selected in each of the four disease categories from the entire sample, matching frequency distribution on age (within five years), race and gender. Smokers were omitted. A total of 180 subjects were selected (PBI-H (n=60), PBI-G (n=60), PBI-MP (n=30) and PBI-SP (n=30) for these analyses. Each GCF sample was analyzed using the Bioplex instrumentation and reagents supplied by R&D Systems (Minneapolis, MN, USA). This instrument permitted the simultaneous measurement of 16 analytes in each GCF sample with the following mean minimum detection level in pg/mL, as follows: IFNg- 0.31 , IL-17- 0.39, IL-1b- 0.27, IL-1ra- 2.06, IL-17 0.39, RANTES- 1.08, MCP-1- 0,95, MIP-1b- 2.12, ENA-78- 2.71, IL-4 - 1.75, IL-5- 0.33, IL-6-0.36, IL-8-0.39, TNFa-0.47, C-SCF- 0.57, IL-10- 0.13 and MIP-1a- 3.10. Mean values and standard errors are shown for only those GCF sample analytes that were both within the range of the assay and also showed significant differences in levels comparing the four groups. Samples with mediator levels that were below the detection level or out of range exceeding the maximum were omitted from these analyses. The mean values for each mediator were adjusted for age, gender and diabetes, and statistical differences between groups tested using SAS GLM proc with a p value of less than 0.05 considered significant.

Plaque and Serum Antibody Assessments

Plaque samples and serum antibody titers were determined using methods recently described by Beck et al (2005). One plaque sample was used from each subject sampling the subgingival mesiobuccal site of the maxillary right first molar. Levels of the eight periodontal pathogens were determined by DNA whole chromosomal checkerboard arrays (*Porphyromonas gingivalis (P. gingivalis), Treponema denticola (T. denticola), Tanarella forsythensis (T. forsythensis), Campylobacter rectus (C. rectus), Prevotella intermedia (P. intermedia), Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans)* and *Fusobacterium nucleatum (F. nucleatum)*). Levels of organism were expressed as CFU equivalents using known microbial standards. Serum IgG levels were determined using immunocheckerboard arrays using these same seven organisms as whole-cell antigens and expressed as ng/ml IgG specific titers using Protein A- bound IgG as an internal standard. For the purposes of this analysis both the level of the organisms and the titer were expressed as high- being above the median and low- being below the median. For descrip-

tive purposes, principal component analyses were performed using SAS, as described by Beck et al (2005).

RESULTS

Serum Antibody and Microbial Loads

Fig 1 displays a principle components analysis exploring the relationship between the four disease groups, relative to the serum antibody IgG level for each organism and the level of organism present. Both the serum antibody and level of pathogen were dichotomized as high or low relative to the median. This diagram reflects the Euclidian distances between the 4 disease categories relative to the low and high antibody titers (ABH, ABL) vs the low and high organism level (BUGH, BUGL). First, it is noteworthy that the organisms and the titers all cluster to form four distinct domains- Low antibody-Low organism, Low antibody-High organism, High antibody-Low organisms and High antibody-High organism. Although there are four regions defined, the organisms and the antibody responses cluster together to form these four tight clusters without any single organism level or antibody response posing as an outlier. Thus the overall level of challenge and quality of the response seems to be consistent across the different organisms within the biofilm.

In Fig 1 it can be seen that (PBI-H) healthy individuals are close to the center of the horizontal axis (organism level axis) indicating that they have neither high levels of organism nor low levels, but they are shifted to the left of center (antibody axis) indicating that they have low antibody responses. The two groups of gingivitis and moderate periodontitis (PBI-G and PBI-MP) appear very close to each other in the centroid indicating that they are not associated with either high or low levels of either IgG or subgingival organism. In contrast, the PBI-S group is shifted into quadrant two indicating that this group is characterized by both a high IgG level and a high level of organism. This is consistent with the concept that the microbial challenge and the antibody response are greater in this group of subjects as compared to the other two diseased groups and much greater than the PBI-H group that have both low organism and low antibody levels .

In Table 1 the GCF level of several inflammatory mediators is shown. For these comparisons non detectible values were not included or averaged into mean values. In Table 1, IL-1b levels show a trend for being progressively higher in the gingivitis, moderate and severe periodontitis groups. The GCF-IL-1b level is significantly higher in the PBI-M group as compared to the healthy group and the level in the PBI-SP group is statistically higher, as compared to all three groups. The GCF level of PGE2 is also significantly elevated in the PBI-SP group relative to the PBI-M group, p=0.03. The
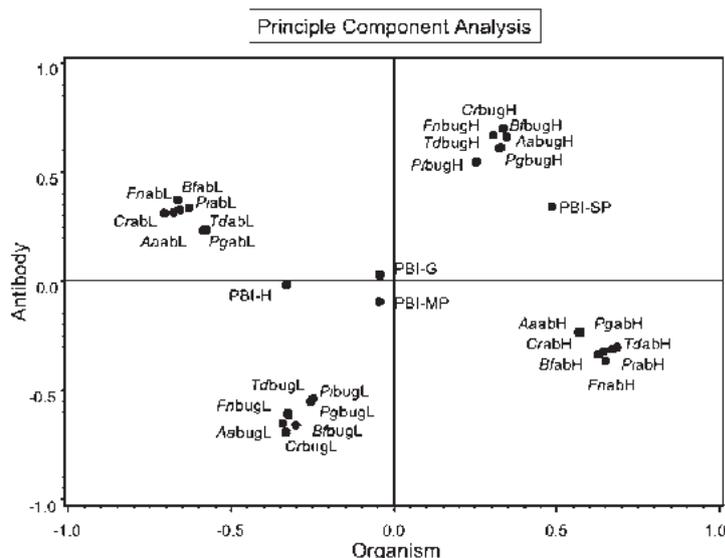
Fig 1 Principle Component Analysis. The Principle Component Analyses SAS output exploring the relationship between periodontal disease classification, bacterial-specific IgG antibody titer, and level of organism for 1673 individuals is shown. PBI-H is the periodontal tissue interface-healthy group, with similar abbreviations for G (gingivitis), MP (moderate periodontitis) and SP (severe periodontitis). The organism abbreviations used are aa-*Actinobacillus actinomycetemcomitans*, pg-*Porphyromonas gingivalis*, pi-*Prevotella intermedia*, cr-*Campylobacter rectus*, bf-*Bacteriodes forsythus* (currently *T forsytheses*) and td-*Treponema denticola*. BUGH and BUGL indicate that the level of the organism is at or above, or below the median respectively. ABH and ABL are abbreviations reflecting that the IgG titer is at or above, or below the median respectively.
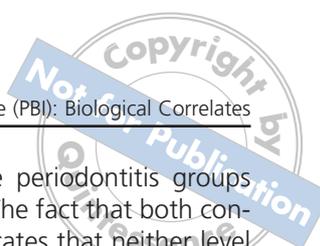
Table 1 GCF Mediators in pg/ml for four PBI groups

GCF Mediators	PBI-H (n=60)		PBI-G (n=60)		PBI-MP (n=30)		PBI-SP (n=30)	
	mean	SE(n)	mean	SE(n)	mean	SE(n)	mean	SE(n)
IL-1β	237.3	43.7(46)	333.5	43.7(45)	397.3[a]	53.1(30)	566.3[b]	57.7(27)
PGE$_2$	196.6	25.5(46)	261.4	24.8(47)	185.4	31.6(29)	300.9[c]	34.0(27)
MIP-1β	29.2	5.8(46)	46.4[d]	5.8(45)	26.1	7.1(30)	35.6	7.7(27)
MCP-1	4.3	1.7(46)	8	1.7(45)	4.6	2.1(30)	10.2[e]	2.3(27)
IL-6	1.42	0.77(44)	2.6	0.76(44)	1.4	0.92(29)	4.54[f]	1.03(24)
ENA78	83.7	8(44)	77.8	7.8(45)	54.3[g]	9.5(27)	72.7	10.3(27)

[a] P=0.02 vs PBI-H
[b] P<0.0001 vs PBI-H, P=0.002 vs PBI-G, P=0.03 vs PBI-MP
[c] P=0.016 vs PBI-H, P=0.013 vs PBI-MP
[d] P=0.04 vs PBI-H, P=0.03 vs PBI-MP
[e] P=0.04 vs PBI-H
[f] P=0.019 vs PBI-H, P=0.02 vs PBI-MP
[g] P=0.019 vs PBI-H

MIP-1b level in the gingivitis group is elevated, as compared to the healthy group and the PBI-MP group, but not different than the PBI-SP group. The GCF-IL-6 level is 3.2 fold higher in the severe periodontitis group as compared to the healthy group (p=0.019) and three-fold elevated as compared to the moderate periodonti-

tis group, p=0.02. Thus, both MIP-1b and IL-6 show a trend towards being associated with the high bleeding scores associated with both the gingivitis and severe periodontitis groups. The PBI-MP group shows decreases in the chemokine ENA78 and is significantly lower, as compared to the PBI-H group, p=0.019.

DISCUSSION

These data subdivide clinical status based upon the probing depths and bleeding extent scores without regard for attachment levels with the intent to focus on the two clinical parameters that are associated with the periodontal tissue-biofilm interface. Although not shown in these analyses, including attachment loss and/or gingival indices into explanatory models of disease extent did not improve the overall predictive value of these models and were deemed non-informative. It is interesting that the level of organism did not distinguish between the healthy, gingivitis or moderate periodontitis individuals. Healthy people had lower levels of antibody to the plaque organisms, perhaps a reflection that these individuals did not experience the same magnitude of systemic exposure to the pathogens or failed to mount a high antibody response to the challenge. It is important that the quality of the antibody response can not be determined by these methods, as the method only measures amount of bound IgG for each organism. It is not known whether the antibody response, albeit a low IgG titer, may be highly opsonic and bacteriocidal and thereby confers protection. By whatever mechanism, health is associated with low total IgG titers. The distinguishing characteristic between gingivitis and moderate periodontitis, as compared to health does not appear to be due to the emergence of any specific biofilm organism. All pathogens appear elevated relative to health, and the serum IgG response is increased in tandem with this load. There were no quantitative differences in IgG that could discriminate between gingivitis or moderate periodontitis in these analyses. Thus, it would appear that the biofilm burden and the systemic antibody response increase in tandem in these two conditions relative to health with no obvious differences in responsiveness as far as the organism-antibody axis is concerned. In contrast, however, severe disease is associated with an increase in both the level of organism and the level of serum IgG. Interestingly, the closest microbial component to the PBI-SP group is the Hi level of *P. gingivalis*. The closest IgG is also *P. gingivalis*-specific. However, the other organisms follow close in close proximity behind this key pathogen. This is generally consistent with the considerable body of evidence linking high levels of *P. gingivalis* and also high serum antibody titers to this organism with severe disease (for review see Whitney et al, 1992). It is interesting in Fig 1

that the gingivitis and moderate periodontitis groups are in such close approximation. The fact that both conditions are near the centroid indicates that neither level of organism or antibody response can effectively discriminate between these two conditions. It is significant that the GCF inflammatory responses, however, can discriminate between these two conditions. Gingivitis has higher MIP-1b as compared to moderate periodontitis. Both are easily distanced from health along the antibody axis, having higher titers, but do not differ with regards to plaque load.

Based upon these relationships one might suggest that serum diagnostics may have the potential to discriminate between severe periodontitis and other periodontal conditions and identify healthy vs diseased. However, it would not help distinguish gingivitis subjects from moderate periodontitis subjects. The flora would only provide insight to identify severe periodontitis subjects from all others. This interpretation would represent an over-simplification, as levels of specific microbes may be useful in a diagnostic mode testing certain thresholds for sensitivity and specificity. However, it does demonstrate a trend that indicates that with the threshold set at the median, the level of one organism closely predicts the level of another and that the level of serum IgG to one organism within the biofilm closely parallels the IgG response associated with another biofilm microbe. It also suggests that the host response to the biofilm is intrinsically different comparing severe periodontitis subjects to all others. And that those that are resistant to disease may also differ. This concept is further supported by the analysis of the GCF.

The increased level of IL-1b within the GCF of diseased subjects closely parallels earlier findings (for review see Champagne et al, 2003). It is important that IL-1b levels are unique in that they distinguish not only between periodontitis and health, but differ in magnitude comparing moderate to severe forms of disease, as well. In contrast PGE2 levels seem to be more associated with high bleeding scores, being highest among both the gingivitis and severe periodontitis groups. The gingivitis groups have no probing depths greater than 3mm, but >10% sites exhibit bleeding on probing. In the PBI-SP group ≥50% exhibit BOP. Thus, this is in general agreement with reports that increased GCF-PGE2 levels may be reflecting the underlying activity of the periodontal lesion and predictive of future attachment loss (Champagne et al, 2003), as BOP is the clinical sign most closely aligned with disease progression. Thus, these findings regarding PGE2 and IL-1b are generally confirmatory in nature. However, the observations regarding the remaining four GCF mediators (MIP-1b, MCP-1, IL-6 and ENA-78) provide a novel perspective on the disease process.
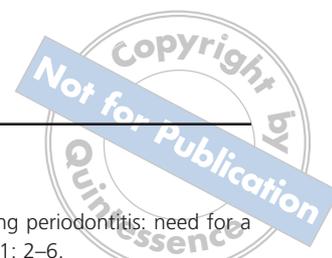
Severe periodontitis is associated with an increase in two key mediators of chronic inflammation IL-6 and MCP-1. IL-6 is critical in that it regulates the transition from acute to chronic inflammation and induces the synthesis of MCP-1. These mediators recruit and activate monocytes and herald the shift from acute to chronic inflammation. IL-6 also is a potent inducer of bone resorption and is selectively elevated in the severe periodontitis group. The fact that IL-6 and MCP-1 are elevated in severe disease and are not significantly increased in either gingivitis or moderate disease (albeit some elevation of MCP-1 in gingivitis) suggests that gingivitis and moderate periodontitis are more alike in terms of the host innate inflammatory response, but that these conditions differ significantly from severe disease. The gingivitis group would appear to be distinguished by a higher level of the chemokine MIP-1b, but caution is needed in interpreting this finding, as it is undetectible in 25% of the subjects. Similarly, moderate periodontitis has depressed levels of the epithelial chemokine ENA-78 relative to all groups, perhaps suggesting an impaired ability to recruit and activate neutrophil defenses in the crevicular environment.

These findings suggest that severe periodontitis at the PBI is associated with a qualitatively different inflammatory response than that associated with gingivitis or moderate periodontitis. Although not shown in this report, principle component analyses that incorporate either microbial levels or plaque scores do not significantly modify this association. The data suggest that individuals with severe periodontitis have higher GCF levels of IL-1b, PGE2, MCP-1 and IL-6. It is not known whether specific gene polymorphisms either individually or in combinations can explain these differences in the innate inflammatory response. This is a strong possibility as functional SNPs which modulate the bioavailability of these mediators have been identified. Thus, genetic variability resulting in Cox-2 ,IL-6 and IL-1 polymorphisms may be an underlying trait which could account for some of these observed differences in disease expression that are associated with severe periodontitis. Considering the wide array of biological variables considered in these analyses, differences in the host innate inflammatory response seem to provide significant information that discriminates between disease states and may merit further study as potential diagnostic marker candidates.

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