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Antibody responses to *Porphyromonas gingivalis (P. gingivalis)* in subjects with rheumatoid arthritis and periodontitis

Ted R. Mikuls, MD, MSPH[*], **Jeffrey B. Payne, DDS, MDS**[†], **Richard A. Reinhardt, DDS, MS, PhD**[†], **Geoffrey M. Thiele, PhD**[*], **Eileen Maziarz, MD**[*], **Amy C. Cannella, MD**[*], **V. Michael Holers, MD**[‡], **Kristine A. Kuhn, MD**[‡], and **James R. O'Dell, MD**[*]

[*]Department of Medicine, Nebraska Arthritis Outcomes Research Center (NAORC), University of Nebraska Medical Center and Omaha Veterans Affairs Medical Center (Omaha, NE, USA)

[†]College of Dentistry, University of Nebraska Medical Center (Lincoln, NE, USA)

[‡]Department of Medicine, University of Colorado Denver School of Medicine (Denver, CO, USA)

Abstract

Summary—Antibody titers to *P. gingivalis* are increased in patients with rheumatoid arthritis and are associated with disease-specific autoimmunity.

Background—Periodontitis (PD) has been implicated as a risk factor for rheumatoid arthritis (RA). We sought to characterize antibody titers to *P. gingivalis* (a pathogen in PD) in subjects with RA, PD, and in healthy controls and to examine their relationship with disease autoantibodies.

Methods—*P. gingivalis* antibody was measured in subjects with RA (n = 78), PD (n = 39), and in controls (n = 40). Group frequencies of bacterial titer elevations were compared using the Chi-square test and antibody titers were compared using non-parametric tests. Correlations of *P. gingivalis* titer with C-reactive protein (CRP), antibody to cyclic citrullinated peptide (anti-CCP), and rheumatoid factor (RF) were examined in those with RA while CRP and autoantibody concentrations were compared based on seropositivity to *P. gingivalis*.

Results—Antibody titers to *P. gingivalis* were highest in PD, lowest in controls, and intermediate in RA ($p = 0.0003$). Elevations in *P. gingivalis* (titer ≥ 800) were more common in RA and PD (67% and 77%, respectively) than in controls (40%) ($p = 0.002$). In RA, there were significant correlations with *P. gingivalis* titer with CRP, anti-CCP-IgM, and -IgG-2. CRP ($p = 0.006$), anti-CCP-IgM ($p = 0.01$) and -IgG2 ($p = 0.04$) concentrations were higher in RA cases with *P. gingivalis* titers ≥ 800 compared to cases with titers < 800.

Conclusion—Antibodies to *P. gingivalis* are more common in RA subjects than controls, although lower than that in PD. Associations of *P. gingivalis* titers with RA-related autoantibody and CRP concentrations suggests that infection with this organism plays a role in disease risk and progression in RA.

Keywords

periodontitis; rheumatoid arthritis; *Porphyromonas gingivalis*; anti-CCP; rheumatoid factor

Corresponding Author: Ted R. Mikuls, MD, MSPH, Associate Professor, Department of Medicine, Section of Rheumatology and Immunology, 986270 Nebraska Medical Center, Omaha, NE, USA 68198-6270; phone (402) 559-7288; fax (402) 559-2466; email: tmikuls@unmc.edu (email and fax number can be published).

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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease with its primary manifestation in the joints, a condition characterized by substantial morbidity and accelerated mortality (1-4). Over the last two decades, the interrelationship between RA and periodontitis (PD) has become increasingly appreciated (5). Several studies have shown an increased frequency of periodontitis (PD) in patients with RA compared to individuals without RA (6-9). It also has been found that measures of RA disease severity (e.g. a higher number of swollen joints, increased C-reactive protein concentration and erythrocyte sedimentation rate) are associated with increased periodontal bone loss (10). Subjects referred for PD treatment are substantially more likely than healthy controls to self-report a diagnosis of RA (11). Even younger patients with recent-onset RA and those with juvenile idiopathic arthritis (JIA) have been reported to have a higher percentage of sites with deep periodontal pockets and periodontal bone loss than controls (12). The association of PD with RA appears to be independent of other risk factors including cigarette smoking, socioeconomic status, body mass index, alcohol consumption, and poor oral hygiene (8).

While most studies investigating the possible etiologic relationship of RA and PD have focused on shared inflammatory mechanisms, there has been limited attention given to bacterial infections that act not only as a primary initiator of PD, but may also play a role in peptide citrullination (the post-translational modification of arginine residues to citrulline residues by peptidylarginine deiminase [PAD]). This is extremely important given the specificity of autoantibody binding to cyclic citrullinated peptides (anti-CCP antibody) in RA (13). Indeed, anti-CCP antibody is associated with disease severity (14,15) in RA and has been postulated to play a pathogenic role in the disease process (16). *Porphyromonas gingivalis (P. gingivalis)* is a gram-negative anaerobic bacterium that is recognized to be a major pathogenic organism in PD and is the only bacteria known to express a PAD enzyme (17). Although not completely homologous to human PAD, similar to its human counterpart this enzyme is responsible for the post-translational conversion of arginine to citrulline. The ability of *P. gingivalis* to express PAD suggests that infection with this organism could impact RA onset and progression by facilitating autoantigen presentation and the expression of disease-specific autoantibody targeting citrullinated peptides, antibody responses that have been shown to be nearly exclusive to RA patients (18).

In this study, we sought to confirm prior observations showing a higher prevalence and concentration of antibody to *P. gingivalis* in RA compared to healthy controls, while also comparing these antibody titers to those with PD. Additionally, we sought to examine the association of antibody directed against *P. gingivalis* with RA-specific autoantibody expression, specifically the presence of anti-CCP antibody and rheumatoid factor (RF) isotypes.

Methods and Materials

Study subjects

We examined banked serum samples collected at baseline from 78 RA patients enrolled in previous randomized clinical trials (19-22). PD status, based on either self-report or clinical probing results, was not known for RA cases. All RA patients satisfied American College of Rheumatology (ACR) classification criteria (23). PD subjects (n = 39) were identified from a pool of patients undergoing periodontal maintenance therapy (regular cleanings) for moderate to severe chronic PD. Serum samples were obtained at the time of evaluation, and the diagnosis of PD was made based on at least two periodontal pockets ≥ 5 mm, defined by clinical probing, and alveolar bone loss identified on bitewing radiographs. PD subjects were otherwise in good general health and none of these subjects reported a diagnosis of RA. In addition to subjects

with RA and PD, we enrolled 40 healthy controls from a pool of available volunteers. Healthy controls were matched to RA cases based on age and sex. Controls were excluded if they self-reported RA or had received tetracycline therapy (a treatment option for PD and/or RA) within the previous six months. All study subjects were ≥ 19 years of age and provided informed written consent for study participation. The protocol was approved by the Institutional Review Board (IRB) at the University of Nebraska Medical Center.

Smoking history (never, former, current) was obtained at the time of enrollment for all healthy controls and subjects with PD. Because smoking history was not routinely assessed on RA patients at the time of clinical trial enrollment, serum cotinine (a byproduct of nicotine) was measured as a surrogate marker of `current' smoking status using a commercially-available ELISA (Institute of Cancer Prevention, Valhalla, New York). RA subjects were considered to be current smokers if serum cotinine concentration was ≥ 100 ng/ml. The utility of this classification was examined in 30 separate RA cases with definitive cotinine values (0 ng/ml or ≥ 100 ng/ml) in whom smoking history was available (11 smokers, 19 non-smokers), calculating a kappa coefficient as a measure of agreement between smoking history (current vs. past or never) and cotinine category (high vs. undetectable serum concentration). Based on the interpretation criteria proposed by Landis and Koch (24), there was `near-perfect' agreement between these measures (kappa = 0.93).

Antibody to P. gingivalis

Strain 381 of *P. gingivalis* was grown as previously described in reducing broth (10 g of yeast extract, 30 g of Trypticase soy broth, 1 g cysteine, 100 mg of dithiothreitol (DTT), 5 mg of hemin and 2.5 mg of menadione in a 1-liter volume) (25). The cells were grown with constant low-speed shaking (150 rpm) at 37°C for 24 hours to an optical density of 1.5 at 660 nm. Sonic extracts of *P. gingivalis* were prepared as described by Tsai et al (26). An enzyme-linked immunosorbent assay (ELISA) was adapted from the procedure described by Engvall and Perlmann (27). The *P. gingivalis* antigen was coated to ELISA plates using 100-200 ng/well of sonic extracts. Two-fold serum dilutions from patients (first dilution 1:100) were added to the plate and the bound human IgG was detected with a peroxidase-conjugated, affinity-purified anti-human IgG (Sigma Chemical Co., St. Louis, MO) followed by a developer containing o-phenylenediamine (OPD) (Becton-Dickinson, Palo Alto, CA). The final dilution is reported as the highest dilution that had an optical density two-times over background (no human serum added). The *P. gingivalis* titer was defined as the inverse value of the largest serial dilution for which detectable antibody was observed. Serial dilutions were carried out to a maximum of 1:204,800. All *P. gingivalis titers* > 204,800 were truncated and assigned 204,800 for the analysis. *P. gingivalis* antibody seropositivity was established as values ≥ 800 (the lowest titer that was more than 2 standard deviations above mean of the log-transformed titer in healthy controls from this study).

Laboratory measures in RA patients

C-reactive protein (CRP, mg/L, Alpha Diagnostic International, San Antonio, TX) and total IgG second generation anti-CCP antibody (U/ml, Axis-Shield Diagnostics, UK) were measured using ELISA. Anti-CCP antibody isotypes (U/ml) were determined by substitution of the conjugate antibody with an isotype-specific antibody (IgM, IgA, IgG1, IgG2, IgG3, or IgG4; Caltag, Burlingame, CA) conjugated to alkaline phosphatase and diluted 1:500 - 1:1000 in PBS. The standard used for determination of U/ml antibody reactivity was that provided by the manufacturer regardless of isotype measured. RF isotypes (IU/ml) were measured using commercially-available ELISAs for IgM, IgA, and IgG (Inova, San Diego, CA, USA). Total anti-CCP antibody (IgG) and RF-IgM were considered to be positive in RA subjects with serum concentrations ≥ 5 U/ml and 9.5 IU/ml (28), respectively. Given our focus on isotype responses specific to RF, total RF (by nephelometry) was not measured. HLA-DRB1 shared epitope (SE)

containing alleles were genotyped as previously reported (29). RA subjects were categorized as SE positive (1 or 2 copies) or negative (0 copies). In addition to the above measures on RA subjects, we also measured anti-CCP antibody (total IgG) on samples of patients with PD.

Statistical analysis

Subject characteristics were compared by group (RA, PD, and controls) using the ANOVA and the non-parametric Wilcoxon Rank sum test for continuous variables and the Chi-square test for all categorical variables (Fisher's Exact test when appropriate). *P. gingivalis* titers were compared by group using the Kruskal-Wallis test and pairwise tests were performed using Wilcoxon rank sum test. The frequency of *P. gingivalis* seropositivity (titer ≥ 800) was compared by group (and in pairwise comparisons) using the Chi-square test.

In analyses limited to RA subjects (n = 78), the associations of *P. gingivalis* titer with CRP, RF, and anti-CCP antibody isotypes were examined using Spearman rank correlation. We also compared concentrations of CRP, RF, and anti-CCP antibody isotypes based on evidence of elevated antibodies to *P. gingivalis* (individuals with titer ≥ 800 vs. < 800) using the non-parametric Wilcoxon rank sum test. To explore whether other factors might explain differences in autoantibody concentration based on evidence of seropositivity to *P. gingivalis*, we also compared age, gender, and the frequency of current smoking by group (RA subjects with higher levels of *P. gingivalis* antibody vs. subjects with lower antibody concentrations) using non-parametric tests for continuous variables and the Chi-square test for dichotomous variables. Statistical analyses were performed using SAS v9.1 (SAS Inc, Cary, NC) and STATA v10 (College Station, TX).

Results

Characteristics of study subjects are summarized in Table 1. Subjects were predominantly Caucasian and were similar across groups with regards to gender and age, although individuals with RA and PD were more likely than healthy controls to be current smokers. Among RA subjects, 91% were seropositive for anti-CCP antibody (as measured using total IgG) and 92% were seropositive for RF-IgM. In contrast, only two of 39 subjects with PD (5%) were seropositive for anti-CCP antibody.

Median *P. gingivalis* antibody titers are summarized for each study group in Table 1. Median antibody titers to *P. gingivalis* were highest in subjects with moderate to severe PD, lowest in healthy controls, with RA subjects having intermediate values ($p = 0.0003$ for differences in medians across groups; $p < 0.02$ for all pair-wise comparisons). Forty percent of healthy controls had evidence of immunity to *P. gingivalis* (titer ≥ 800), compared to 67% of those with RA ($p = 0.006$ RA vs. controls) and 77% for those with PD ($p = 0.0009$ PD vs. controls) ($p = 0.002$ across groups).

In analyses limited to RA subjects (n = 78), there were significant correlations of *P. gingivalis* titer with C-reactive protein (CRP) ($r = 0.33$, $p = 0.003$), anti-CCP-IgM ($r = 0.27$, $p = 0.02$), anti-CCP-IgG-2 ($r = 0.29$, $p = 0.01$), and anti-CCP-IgG-4 ($r = 0.23$, $p = 0.05$). Associations of P. gingivalis titers with total anti-CCP antibody ($r = 0.21$, $p = 0.06$) and anti-CCP-IgG-1 ($r = 0.20$, $p = 0.08$) were borderline, not meeting the threshold for statistical significance (Table 2). In contrast, there were no associations of *P. gingivalis* concentrations with anti-CCP-IgA or -IgG-3 and there were no associations with any of the RF isotypes. CRP (median 10.8 vs. 7.4 mg/L, $p = 0.006$), anti-CCP-IgM (median 7.9 vs. 3.1 U/ml, $p = 0.01$) and - IgG2 (median 2.9 vs. 2.1 U/ml, $p = 0.04$) antibody concentrations were higher in those with *P. gingivalis* titers ≥ 800 compared to those with lower titers (Table 3). Although not reaching statistical significance, there was a trend toward higher concentrations of total IgG anti-CCP antibody in those seropositive for *P. gingivalis* compared to those with titers below 800 (median

221 vs. 135 U/ml, p = 0.09). In an examination of other patient factors, there were no differences in age, gender, current smoking status, or SE positivity based on seropositivity to *P. gingivalis*.

Discussion

In this study, we found that both the prevalence and magnitude of antibody responses to *P. gingivalis* was greater in RA cases compared to healthy controls, although less than that observed in subjects with established and moderate to severe PD. With reports suggesting an overall prevalence of PD approaching 10-20% (30), the relatively high frequency of seropositivity to *P. gingivalis* in controls suggests that exposure and subsequent antibody responses to this bacterium may be more common then clinically relevant PD. To our knowledge, this is the first study to show an association of *P. gingivalis* antibody concentrations with select anti-CCP antibody isotypes (most notably anti-CCP-IgM and -IgG2 subclass), important given the specificity of anti-CCP antibody in RA (13) and its potential pathogenic role in the disease (31). The association of this infection with select anti-CCP antibody isotypes and CRP concentrations suggests that infection with this bacterium may play a role in some patients with RA, affecting both adaptive and non-specific inflammatory pathways. The association of immune responses to *P. gingivalis* with anti-CCP-IgG2 subclass concentrations may be particularly noteworthy since the IgG2 subclass represents the predominant serum IgG response to infection with this organism (32). The lack of difference in anti-CCP-IgA based on seropositivity to *P. gingivalis* was not expected given the mucosal nature of this infection in addition to a prior report showing significant IgA antibody responses to this infection in chronic PD (33). The relationship of *P. gingivalis* infection with higher CRP concentrations also confirms reports examining the impact of PD on acute phase responses in RA (10) Given the limited sample size coupled with the expectation of low levels of P. gingivalis antibody response and lower systemic inflammation, we did not examine associations of *P. gingivalis* titers with CRP concentrations in controls.

We cannot conclude from these findings alone whether infection with *P. gingivalis* serves as a trigger for RA onset, or alternatively acts to modify immune responses in subjects following disease onset, or both. It has been hypothesized that humoral immune responses to oral bacteria, namely *P. gingivalis*, provide a potent stimulus for the development of RA in select cases (34). This hypothesis is attributed, at least in part, to several shared pathophysiologic mechanisms between RA and PD including: 1) similar inflammatory and histopathologic disease characteristics (35), 2) shared environmental and genetic risk factors (such as HLA-DR4-subtypes 0401, 0404, 0405, 0408) (36), and 3) overlapping treatment strategies, including metalloproteinase inhibition, (37) in addition to pre-clinical data supporting the role of newer RA-approved therapies in the treatment of PD (agents targeting both tumor necrosis factor [TNF]α and interleukin [IL]-1) (34,38,39).

As noted by Rosenstein and colleagues (34), *P. gingivalis* is the only prokaryotic organism that produces PAD, a virulence factor for this organism (17,40). Although functionally distinct from mammalian forms of this enzyme, bacterial PAD has the capacity of deiminating arginine in fibrin found in the periodontal lesion (40). Similar to its bacterial counterpart, human PAD expressed in the joint has the capacity to deiminate synovial fibrin and, with epitope spreading, these synovial antigens may serve as targets for autoantibody formation triggered by oral infections associated with PD. This is especially noteworthy since citrullinated variants of the α- and β-fibin chains have been proposed as target `autoantigens' in the rheumatoid joint (41). This has led to speculation that `individuals predisposed to (PD) are exposed to citrullinated antigens that, in the proinflammatory context of PD, become systemic immunogens' (34).

In contrast to associations with anti-CCP antibody, we found no association of immunity to *P. gingivalis* with RF isotype concentrations in patients with RA. This contrasts with reports showing that RF can be detected in the gingiva, subgingival plaque, and serum of patients with PD (42,43). Locally-produced RF in PD appears to be most closely linked to infections with *Fusobacterium nucleatum* and *Capnocytophaga ochracea*, oral pathogens that do not express PAD (42,44). With lower disease specificity, it has been suggested that RF serves primarily to `amplify' inflammatory responses both in the rheumatoid joint (45) as well as the lesion in PD (46), rather than having a direct role in disease pathogenesis. It is possible that these null findings result from its lower specificity and our inability to distinguish smaller differences in RF expression with a limited sample size, particularly in light of the background seropositivity in this cohort with enrollment criteria including RF positivity (20,21).

There are other limitations to this study. Given the `retrospective' nature of the RA cases and corresponding samples, formal periodontal evaluations were not available for this group. It is also possible that differences in antibody responses to *P. gingivalis* based on disease status may be due to other factors (e.g., cigarette smoking). Current smoking was much more frequent in subjects with RA and PD compared to healthy controls, which is important given separate associations of smoking with both PD and RA risk (47-49). The small number of healthy controls (n = 2) self-reporting current smoking precluded adjustment for this variable in our comparisons by disease status. The lack of self-reported smoking data among RA cases also prohibited analyses examining differences in former or ever smoking. However, it is worth noting that others have shown that the association of PD with RA appears to be independent of underlying smoking status (10). Furthermore, it is unlikely that variation in current smoking status explained the observed differences in autoantibody concentrations in the analysis limited to RA subjects (case only comparisons based on *P. gingivalis* seropositivity) with there being nearly identical frequency of current smokers among comparator groups. Our study was limited primarily to Caucasian women, suggesting that our results may not be generalizable to other populations. As noted, the cross-sectional design of this study limits any causal inferences that can be made regarding the association of *P. gingivalis* infection and RA onset. Future studies are needed to replicate these findings in larger and more diverse patient populations and to explore the potential biologic link between *P. gingivalis* infection and other oral infections with incident RA, disease severity, and autoantibody expression. Intervention studies aimed at identifying and reducing the level of *P. gingivalis* infection in the context of RA, coupled with comprehensive assessments on disease course and clinical outcomes, could be helpful.

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Table 1

Subject characteristics and evidence of immunity to *P. gingivalis* by disease status: a comparison of subjects with rheumatoid arthritis (RA), healthy controls, and subjects with moderate to severe periodontitis (PD); values shown are number (%) or median (range)

	RA (n = 78)	Controls (n = 40)	PD (n = 39)	P-value[†]
		% or Median (Range)		
Women	64 (78%)	31 (78%)	29 (74%)	0.96
Age	49 (24-79)	49 (21-76)	52 (33-61)	0.82
Current smoking[*]	24 (31%)	2 (5%)	16 (41%)	0.0007
Caucasian	42 (89%)	36 (90%)	39 (100%)	0.10
P. gingivalis titer (inverse titer)	800 (0-204,800)	200 (0-204,800)	12,800 (0-204,800)	0.0003
P. gingivalis titer ≥ 800	52 (67%)	16 (40%)	30 (77%)	0.002

[*] "Current smoking" in RA subjects based on serum cotinine concentration (current smoker >= 100 ng/ml, non-smokers with undetectable serum cotinine,; smoking status missing for 1 RA subject); smoking status in control and PD groups were based on self-report by study subjects; race/ethnicity missing for 31 RA subjects.

[†] Group comparisons using one-way ANOVA for age, Chi-square test (Fisher's Exact test where appropriate) for dichotomous variables, and Kruskal-Wallis (non-parametric) test for *P. gingivalis* concentrations; titers truncated at 204,800 and values exceeding this threshold assigned 204,800 for analysis

Table 2

Correlations of *P. gingivalis* antibody (IgG) concentration with C-reactive protein and disease-related autoantibody in RA subjects; Spearman correlation coefficients and corresponding p-values (n = 78)*

	Correlation coefficient (r)	P-value
C-reactive protein (CRP)	0.33	0.003
Anti-CCP-IgM	0.27	0.02
Anti-CCP-IgA	0.03	0.81
Anti-CCP-IgG (total)	0.21	0.06
Anti-CCP-IgG1	0.20	0.08
Anti-CCP-IgG2	0.29	0.01
Anti-CCP-IgG3	0.15	0.20
Anti-CCP-IgG4	0.23	0.05
RF-IgM	0.12	0.28
RF-IgA	0.10	0.38
RF-IgG	0.19	0.10

*CCP = cyclic citrullinated peptide; RF = rheumatoid factor

Table 3

Associations with immunity to *P. gingivalis* in subjects with rheumatoid arthritis: patient characteristics and serum concentrations of C-reactive protein (CRP) and disease-related autoantibody based on evidence of immunity to *P. gingivalis* (n = 78); values shown are percentage or median (range)

	***P. gingivalis* titer ≥ 800 (n = 52)**	***P. gingivalis* titer < 800 (n = 26)**	**P-value**
Disease characteristic			
Age, years	49 (24-79)	51 (28-77)	0.72
Female	75%	84%	0.37
Current smoking	31%	32%	0.91
SE positive	81%	76%	0.57
Serum measure			
CRP, mg/L	10.8 (0.9-89.1)	7.4 (0.1-88.0)	0.006
Anti-CCP-IgM, U/ml	7.89 (0.61-113.5)	3.09 (0.88-96.18)	0.01
Anti-CCP-IgA, U/ml	5.82 (1.69-47.6)	5.53 (1.42-25.25)	0.56
Anti-CCP-IgG1,U/ml	3.88 (0-13.61)	2.40 (0-12.59)	0.14
Anti-CCP-IgG2, U/ml	2.92 (0.70-29.29)	2.10 (0.63-9.94)	0.04
Anti-CCP-IgG3, U/ml	1.86 (0.30-13.44)	1.59 (0.25-8.28)	0.23
Anti-CCP-IgG4, U/ml	2.14 (0-30.83)	0.60 (0-20.17)	0.11
Anti-CCP (total IgG), U/ml	221 (0.78-2626)	135 (0.81-1303)	0.09
RF-IgM, IU/ml	91.8 (0.07-503.0)	73.6 (0.45-503.2)	0.54
RF-IgA, IU/ml	74.0 (0-408.0)	43.3 (2.1-408.0)	0.35
RF-IgG, IU/ml	22.0 (0-510.4)	10.4 (0-338.1)	0.41

SE = HLA-DRB1 shared epitope; CCP = cyclic citrullinated peptide; RF = rheumatoid factor; CRP = C-reactive protein