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Actinobacillus actinomycetemcomitans and *Porphyromonas gingivalis* subgingival presence, species-specific serum immunoglobulin G antibody levels, and periodontitis disease recurrence

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Background and Objective: The biological and clinical effects of antibody against periodontal pathogenic bacteria are incompletely understood. This study evaluated the inter-relationships among periodontal levels of cultivable *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, species-specific serum immunoglobulin G (IgG) antibody levels, and periodontitis disease activity.

Material and Methods: Forty-three adults who had previously been treated for periodontitis and who also harbored cultivable *A. actinomycetemcomitans* or *P. gingivalis* were evaluated semiannually for clinical disease recurrence over a 36-month period. Each patient provided subgingival microbial samples, for the recovery of *A. actinomycetemcomitans* and *P. gingivalis*, from the two deepest pockets in each dentition sextant. *A. actinomycetemcomitans* and *P. gingivalis* serum IgG antibody levels were assessed using enzyme-linked immunosorbent assay (ELISA), together with whole-cell sonicate extracts from *A. actinomycetemcomitans* serotypes a–c and *P. gingivalis* ATCC 33277. Data were analyzed using the Mantel–Haenszel chi-square and Fisher exact two-tailed tests.

Results: Eighteen (60.0%) of 30 *A. actinomycetemcomitans*-positive subjects, and 10 (76.9%) of 13 *P. gingivalis*-positive subjects, exhibited recurrent periodontal breakdown within 36 months of periodontal therapy. Nineteen (67.9%) of the 28 patients with active periodontitis had *A. actinomycetemcomitans* or *P. gingivalis* serum antibody levels below designated threshold values. In comparison, 10 (66.7%) of 15 culture-positive clinically stable subjects showed *A. actinomycetemcomitans* or *P. gingivalis* serum antibody levels above threshold values. The difference between specific antibody levels in periodontitis-active and periodontitis-stable patients was statistically significant ($p = 0.032$).

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Conclusions: Serum levels of IgG antibodies against *A. actinomycetemcomitans* or *P. gingivalis* in periodontitis-stable patients were higher than those in patients with active periodontitis. The results suggest that elevated levels of IgG antibody against *A. actinomycetemcomitans* and *P. gingivalis* have a detectable protective effect against periodontal infections with these microorganisms.

The periodontal pocket is densely populated with bacteria, making it an important site for host–microbe interactions. Periodontal bacteria stimulate B-cell proliferation, mostly through a classical antigen-specific immune response, but stimulation may also take place through B-cell superantigens or through the innate immune system. Several major periodontal pathogens, including *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, are capable of inducing a robust serum antibody response (1). In fact, individuals infected with these two species of bacteria can exhibit serum antibody levels which exceed those found for many bacteria in medical infections. Particularly high antibody levels against *A. actinomycetemcomitans* are detected in juveniles with localized aggressive periodontitis (2) and against *P. gingivalis* in adults with advanced periodontitis (3). As *A. actinomycetemcomitans* and *P. gingivalis* only seem to inhabit the human oral cavity, the predominant antibody response against these two bacteria is derived from an oral source of infection, probably periodontal sites.

Preventing periodontitis through immunological approaches is appealing, but mechanisms of protective immunity in periodontal tissues are incompletely understood. A recent hypothesis suggests that periodontitis occurs in part because of a genetically determined nonefficacious immune response to periodontopathic bacteria (4,5). Despite decades of research, the relationship between specific serum immunogloblin G (IgG) antibody level and protection against periodontal pathogens, including *A. actinomycetemcomitans* and *P. gingivalis*, remains obscure. Some studies found high antibody levels against these two periodontopathic bacteria to be associated with relative disease stability, whereas other studies detected no discernible antibody effect on disease activity (1). Discrepancy in research findings may be a consequence of using different methods to measure antibody responses and difficulties in assessing periodontitis disease activity. Delineating the role of host immune responsiveness in periodontal disease may provide new approaches for the control of periodontitis through the development of safe and effective vaccines.

To further elucidate the extent to which periodontal pathogens may stimulate systemic immune responses, and to understand how specific antibody responses may affect periodontal disease activity, the present study evaluated the serum IgG antibody response of periodontitis patients to whole-cell sonicates from *A. actinomycetemcomitans* and *P. gingivalis*. This study also aimed to relate periodontitis disease activity to levels of cultivable *A. actinomycetemcomitans* and *P. gingivalis* and to the levels of serum IgG antibodies against the two microorganisms.

Material and methods

Subjects and clinical examinations

The patient population data were obtained from a prospective study of periodontitis disease recurrence. The experimental design, patient selection criteria, examination techniques, and clinical and microbiological findings have been described in detail previously (6,7). In brief, 98 adults, previously treated for moderate to advanced periodontitis, were enrolled in a systematic 3-month maintenance care program, and were clinically evaluated semiannually for disease recurrence over a 36-month period. Recurrent periodontitis disease activity was defined as any site, identified by two independent examiners, which exhibited either a probing depth increase of 3 mm or greater from baseline, or a probing depth increase of 2 mm or greater, together with a loss of relative periodontal attachment level of 2 mm or greater from baseline, as measured from the margin of an occlusal reference stent.

Baseline data on culture recovery of subgingival *A. actinomycetemcomitans* and *P. gingivalis* were compared with baseline serum levels of *A. actinomycetemcomitans* or *P. gingivalis* IgG antibodies in all 98 study subjects. In addition, baseline microbiological and immunological parameters were related to disease recurrence over 36 months in a subset of 43 *A. actinomycetemcomitans* or *P. gingivalis* culture-positive subjects.

Microbiological procedures

As previously described (6), *A. actinomycetemcomitans* and *P. gingivalis* were recovered from subgingival specimens obtained by paper points from the two pockets with the deepest probing depths in each of the study subject's six dentition sextants. Following a 2-h transport in VMGA III (8), and after dispersion using a Vortex mixer, 10-fold serial dilutions of plaque specimens were plated onto nonselective enriched Brucella blood agar (EBBA) for the recovery of *P. gingivalis* and the determination of total anaerobic viable counts (6), onto a selective medium for the isolation of black-pigmented gram-negative anaerobic species (9), and onto TSBV medium for the selective recovery of *A. actinomycetemcomitans* (10). EBBA plates and the selective medium for black-pigmented gram-negative anaerobic species were

incubated at 35°C in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, MI, USA), containing 85% N_2, 10% H_2 and 5% CO_2, for 10 days. TSBV plates were incubated at 35°C in 10% CO_2/90% N_2 for 4 days. Identification of *A. actinomycetemcomitans* and *P. gingivalis* isolates was carried out using the criteria and techniques of Slots (11,12). The proportional recovery of subgingival *A. actinomycetemcomitans* and *P. gingivalis* was determined from their cultivable counts in relation to total anaerobic viable counts on nonselective EBBA plates.

Serum levels of IgG antibodies against *A. actinomycetemcomitans* and *P. gingivalis*

The immunological assays used were as previously described (7). In brief, an enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of IgG antibodies to *A. actinomycetemcomitans* and *P. gingivalis* in serum collected by venepuncture from each of the 98 study subjects. Antigens for ELISA were prepared from whole-cell extracts from *A. actinomycetemcomitans* serotypes a (ATCC 29523), b (Y4) and c (NCTC 9710), and from *P. gingivalis* ATCC 33277, as previously described (7). The coating and processing of ELISA plates, dilution of patient sera, incubation conditions, and determination of the optical density of ELISA plate wells, followed procedures previously described (7). *A. actinomycetemcomitans* and *P. gingivalis* antibody levels were expressed as the area in square millimeters under the dose–response dilution curve, using the formula described by Sedgwick *et al.* (13) and Pussinen *et al.* (14). The 90th percentile of all ELISA *A. actinomycetemcomitans* and *P. gingivalis* antibody measurements, determined by using an Excel 2000 spreadsheet (Microsoft Corp., Redmond, WA, USA), served as a threshold for the identification of study subjects demonstrating high vs. low serum IgG antibody levels to the two study organisms.

All microbiological and immunological laboratory procedures were performed blind, without knowledge of the clinical status of the study subjects or of the periodontal sites sampled.

Data analysis

Odds ratio analysis (15) was used with the Mantel–Haenszel chi-square test (16) or, for cell numbers less than 5, with the Fisher exact two-tailed test, to examine the relationship in subjects between the cultivable subgingival recovery of *A. actinomycetemcomitans* and *P. gingivalis* and the homologous serum IgG antibody level, and to assess the relationship between serum levels of IgG antibodies to *A. actinomycetemcomitans* or *P. gingivalis* and recurrent periodontitis disease activity. Positive predictive values (17) were calculated for the relationship between serum *A. actinomycetemcomitans* or *P. gingivalis* IgG antibody levels and clinical periodontal status over 36 months. The Student's *t*-test was used to determine significant differences in mean cultivable proportional recovery of subgingival *A. actinomycetemcomitans* and *P. gingivalis* between subjects with high- and low-serum *A. actinomycetemcomitans* and *P. gingivalis* IgG antibody levels. A p-value of ≤ 0.05 was required for statistical significance.

Results

Culture analysis revealed the presence of subgingival *A. actinomycetemcomitans* in 31 (31.6%) of the 98 study subjects, and a mean of 2.1% (range: 0.0002–8.1%) *A. actinomycetemcomitans* isolates in culture-positive patients. Subgingival *P. gingivalis* was recovered from 13 (13.4%) study subjects, yielding an average of 3.3% (range: 0.1–16.5%) culturable *P. gingivalis*. No study subject revealed a dual infection of subgingival *A. actinomycetemcomitans* and *P. gingivalis*.

A total of 95 (96.9%) study subjects was found to have serum IgG antibody to one or more of the three *A. actinomycetemcomitans* serotypes tested, with the area under the ELISA curve ranging from 0 to 34.5 mm^2 per subject. Thirty (30.6%) subjects were found to have high serum *A. actinomycetemcomitans* IgG antibody levels to one or more of the three serotypes, using a threshold of 90th percentile and greater ($\geq$ 15.1 mm^2) of all *A. actinomycetemcomitans* ELISA antibody values. High anti-*A. actinomycetemcomitans* antibody levels were most frequently detected against serotype c, either alone (14 subjects), or in joint high occurrence with serotype a (one subject). High IgG antibody levels against *A. actinomycetemcomitans* serotype b occurred in eight subjects and against serotype a in seven subjects.

Forty-two (42.9%) study subjects revealed serum IgG antibody reactivity to *P. gingivalis*, with ELISA antibody values ranging from 0 to 7.2 mm^2 per subject. Eleven (11.2%) subjects showed high serum *P. gingivalis* IgG antibody levels when the 90th percentile and greater ($\geq$ 3.3 mm^2) of all *P. gingivalis* ELISA antibody levels was used as threshold.

Table 1 shows the relationship between subgingival presence and serum IgG antibody levels for *A. actinomycetemcomitans* and *P. gingivalis*.

Table 1. Relationship between the subgingival presence of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* and homologous serum immunoglobulin G (IgG) antibody levels in 98 adults with treated periodontitis

	Culture-positive patients	Culture-negative patients
A. actinomycetemcomitans serum IgG antibody level		
$\geq$ 15.1 mm^2	16[a]	14
$<$ 15.1 mm^2	15	53
P. gingivalis serum IgG antibody level		
$\geq$ 3.3 mm^2	4[a]	7
$<$ 3.3 mm^2	9	78

[a]Number of subjects; IgG, Immunoglobulin G (see Table 2).

Sixteen (51.6%) of the 31 *A. actinomycetemcomitans* culture-positive subjects exhibited a high serum IgG antibody level to one or more of the three *A. actinomycetemcomitans* serotypes tested (ELISA curve threshold area of 15.1 mm^2). Odds ratio analysis revealed a statistically significant positive subject relationship between the cultivable occurrence of subgingival *A. actinomycetemcomitans* and high homologous serum IgG level (odds ratio 4.0; $p = 0.002$, Mantel–Haenszel chi-square test). However, the mean recovery of *A. actinomycetemcomitans* was not significantly different in 16 subjects (standard error: 6.0 ± 1.8%) exhibiting high, and in 14 subjects (5.7 ± 1.9%) showing low, anti-*A. actinomycetemcomitans* serum IgG antibody levels. Four (30.8%) of 13 *P. gingivalis* culture-positive subjects showed a high IgG antibody level to the organism (ELISA curve threshold area of 3.3 mm^2). A statistically significant positive subject association was found between the occurrence of subgingival *P. gingivalis* and a high homologous serum IgG antibody level (odds ratio 5.0; $p = 0.037$, Fisher exact two-tailed test). The mean recovery of *P. gingivalis* was not significantly different in three subjects (1.5 ± 0.4%) demonstrating high, and in 10 subjects (3.9 ± 1.5%) revealing a low, anti-*P. gingivalis* serum IgG antibody level.

Table 2 presents the relationship between *A. actinomycetemcomitans* or *P. gingivalis* serum IgG antibody levels and recurrent periodontitis disease activity in the 43 culture-positive subjects over the 36 study months. Eighteen (60.0%) of the 30 *A. actinomycetemcomitans*-positive subjects and 10 (76.9%) of the 13 *P. gingivalis*-positive subjects experienced recurrent periodontal breakdown in the post-treatment follow-up period. Perhaps, because of the relatively small number of study patients, when *A. actinomycetemcomitans* and *P. gingivalis* data sets were analyzed individually, no statistically significant relationship was found between culture-positive subjects with low serum antibody levels against either of the organisms and periodontitis disease activity (data not shown). However, when the *A. actinomycetemcomitans* and *P. gingivalis* data sets were combined, it was found that among the 28 culture-positive and disease-active subjects, 19 (67.9%) exhibited *A. actinomycetemcomitans* or *P. gingivalis* serum antibody levels below the designated threshold values. Patients who demonstrated cultivable *A. actinomycetemcomitans* or *P. gingivalis*, together with low homologous serum antibody levels, showed a positive predictive value of 79.2 and an odds ratio of 4.2 ($p = 0.032$, Mantel–Haenszel chi-square test) for periodontitis disease recurrence. Also, the difference between specific antibody levels in periodontitis-active and periodontitis-stable patients was statistically significant ($p = 0.032$, Mantel–Haenszel chi-square test).

Table 2 also shows that 10 (66.7%) of the 15 culture-positive subjects, who were clinically stable, revealed *A. actinomycetemcomitans* or *P. gingivalis* serum IgG antibody levels above the designated threshold values. Patients who demonstrated cultivable *A. actinomycetemcomitans* or *P. gingivalis*, together with high homologous serum antibody levels, showed a positive predictive value of 52.6 and an odds ratio of 4.2 ($p = 0.032$, Mantel–Haenszel chi-square test) for exhibiting nonprogressing periodontal disease.

Table 2. Periodontitis recurrence over 36 months in 43 subjects harboring either subgingival *Actinobacillus actinomycetemcomitans* or *Porphyromonas gingivalis*[a]

	Periodontitis disease-active patients	Clinically stable patients
A. actinomycetemcomitans culture-positive patients		
A. actinomycetemcomitans serum IgG antibody levels		
≥ 15.1 mm^2	8[b]	8
< 15.1 mm^2	10	4
P. gingivalis culture-positive patients		
P. gingivalis serum IgG antibody levels		
≥ 3.3 mm^2	1[b]	2
< 3.3 mm^2	9	1
A. actinomycetemcomitans or *P. gingivalis* culture-positive patients		
High *A. actinomycetemcomitans* or *P. gingivalis* serum IgG antibody levels to homologous subgingival organism[c]		
+	9[b]	10
–	19	5

[a]No subject yielded both *A. actinomycetemcomitans* and *P. gingivalis*.
[b]Number of subjects.
[c]Values were ≥ 15.1 mm^2 for *A. actinomycetemcomitans* and ≥ 3.3 mm^2 for *P. gingivalis*.
IgG, immunoglobulin G.

Discussion

Virtually all previous studies on infectious periodontal disease have been limited to examining relationships between bacterial occurrence and periodontitis disease severity (18–20), between bacterial presence and homologous antibodies (21,22), or between specific antibody levels and periodontitis disease severity (2,23–28). Moreover, the design of most previous studies has been cross-sectional (21,29), and some of the few longitudinal studies have included relatively short follow-up periods (30,31). Studies that only examine a few aspects of the complex inter-relationships of bacteria–host resistance–periodontitis may be unable to elucidate critical pathogenic determinants of destructive periodontal disease. To overcome some of the weaknesses of previous

studies, the present investigation simultaneously determined the occurrence of subgingival *A. actinomycetemcomitans* and *P. gingivalis*, systemic IgG antibody levels against the two species in infected subjects, and periodontitis disease activity assessed over a period of 36 months.

Almost one-third of the study patients yielded *A. actinomycetemcomitans*. Although *A. actinomycetemcomitans* is commonly regarded as a pathogen of localized aggressive periodontitis (32), it is also frequently recovered from adult periodontitis lesions that are refractory to conventional mechanical therapy (33). The ability of *A. actinomycetemcomitans* to invade gingival tissue (34) may explain why the organism is largely unaffected by thorough scaling and root planing (35). Despite the importance of *P. gingivalis* in adult periodontitis (33), the organism was cultured from only a few study patients, a finding confirmed by immunofluorescence staining of direct subgingival smears (36). As periodontal *P. gingivalis* is confined to the pocket area, it has the potential to be markedly suppressed or eradicated by thorough mechanical periodontal therapy (35).

The major findings of the present study were the detection of relatively low *A. actinomycetemcomitans* and *P. gingivalis* antibody levels in patients with progressing periodontitis, and of relatively high antibody levels to both species in stable periodontitis patients. Those data are consistent with the notion that specific serum antibody responses towards *A. actinomycetemcomitans* and *P. gingivalis* confer protection for patients at risk of periodontitis. Studies have also shown that immunization with *P. gingivalis* can inhibit the progression of experimental periodontitis in nonhuman primates (37,38), rats (39,40) and mice (41), and can significantly reduce bacterial counts and tissue reactions in inoculated tissue cages of rabbits (42). Antibodies may neutralize toxins of *A. actinomycetemcomitans* (43) and proteolytic enzymes of *P. gingivalis* (44,45), as well as promote opsonization, agglutination/precipitation, steric hindrance of attachment, antibody-dependent cell-mediated cytotoxicity, and complement activation (1). Thus, antibodies may not necessarily need to decrease periodontal pathogen loads in order to exert a protective effect, a notion that may help to explain the present finding of no relationship between specific serum antibody levels and the proportional recovery of the two test microorganisms in patients with stable periodontitis. Parenthetically, the observation that elevated antibody responses against *A. actinomycetemcomitans* and *P. gingivalis* seemed to help prevent further periodontal destruction provides additional evidence of the importance of the two organisms in the pathogenesis of periodontitis.

The finding of a positive relationship between subgingival *A. actinomycetemcomitans* counts and *A. actinomycetemcomitans* IgG antibody levels in serum has been described previously (46). A high pathogen load will generally induce a vigorous host immune response. It is less clear why eight *A. actinomycetemcomitans*-positive patients and one *P. gingivalis*-positive patient experienced continuing periodontal destruction, despite high specific antibody levels. In refractory human periodontitis (47), and in nonhuman primates immunized with *P. gingivalis* antigens (48,49), periodontal disease progression has previously been reported to occur in the presence of high anti-pathogen antibody levels. First, as discussed previously (50), some periodontitis cases may have involved herpesviruses, the control of which is dependent upon cellular immunity rather than upon antibodies. Second, the presence of subgingival microorganisms, other than *A. actinomycetemcomitans* and *P. gingivalis*, may have sustained further periodontal breakdown (20). Third, in spite of high specific antibody levels, the elicited antibody may vary widely in functional capacity (51) and a major portion may have been directed against bacterial antigens of little or no pathogenic relevance rather than towards critical antigens. A novel means of identifying critical virulence determinants in periodontopathic bacteria has recently been described (52). Fourth, the specific serum antibody may have been predominantly of the IgG2 isotype (53), which is mostly elicited by polysaccharide antigens, and which possesses relatively low avidity (54,55) and lacks strong complement fixation and opsonic properties (55,56). There may exist a degree of genetic control over the immunoglobulin phenotype response to *P. gingivalis* (57). Fifth, serum antibodies towards one periodontal pathogen may alter the functional antibody response against another periodontopathic microorganism (58,59). Sixth, some periodontal lesions may have harbored *A. actinomycetemcomitans* or *P. gingivalis* loads too heavy to be effectively controlled by the antibody threshold level used in the present study (60). Seventh, bacterial proteases in periodontal sites may have degraded antipathogen antibodies (61) or the C3 protein (62). Eighth, although less likely, some patients may have experienced periodontitis as a result of antibody-mediated immunopathological reactions (63).

Atypical were also the four culture-positive *A. actinomycetemcomitans* patients and the one culture-positive *P. gingivalis* patient, who exhibited low levels of homologous serum antibody but still did not experience periodontal breakdown during the 36-month study period. As *A. actinomycetemcomitans* and *P. gingivalis* may require a supporting active herpesvirus infection to produce periodontal destruction (50), the stable periodontitis lesions may have been devoid of periodontopathic herpesviruses. The possibility also exists that the infecting *A. actinomycetemcomitans* and *P. gingivalis* strains belonged to a less pathogenic serotype (64,65), or lacked one or more critical virulence factors (66,67). *A. actinomycetemcomitans* serotype c, which evoked a high antibody response in 15 of our study patients, may exert less periodontopathogenicity than serotype b (68,69), perhaps because serotype c strains tend to elicit high-avidity IgG antibodies (70). Finally, the available antibody may have effectively neutralized key virulence determinants and therefore sufficed in protecting the periodontal tissues.

In conclusion, the present study provides evidence that IgG antibodies against *A. actinomycetemcomitans* and *P. gingivalis* exert a protective role in periodontitis. After delineating critical antigens of periodontal pathogenic bacteria, studies are warranted to evaluate the feasibility of developing a vaccine against periodontitis.

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