

Isolation of *Porphyromonas gingivalis* and Detection of Immunoglobulin A Specific to Fimbrial Antigen in Gingival Crevicular Fluid

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The present study evaluated the prevalence of *Porphyromonas gingivalis* and the correlation between the bacterial culture method and the detection of immunoglobulin A (IgA) specific to the *P. gingivalis* fimbrial antigen in gingival crevicular fluid (GCF). *P. gingivalis* was isolated from 78.3% of subgingival plaque samples obtained from active sites and 34.7% of those from inactive sites of periodontal patients. *P. gingivalis* was isolated from only 4.7% of healthy subjects (control group). Immunoglobulins specific to the *P. gingivalis* fimbrial antigen were detected by enzyme-linked immunosorbent assay (ELISA). The overall agreement between the results of the *P. gingivalis* culture method and the results of specific IgA detection in periodontal patients was 71.7% for active sites and 58.7% for inactive sites. IgA specific to *P. gingivalis* was absent in GCF from all of the sites of healthy subjects. The results suggest that *P. gingivalis* is associated with the local production of specific IgA. The detection of IgA antibodies specific to *P. gingivalis* in GCF by ELISA may be used as a predictive parameter to reveal the early phase of the activation of recurrent periodontal infections.

Porphyromonas gingivalis has been frequently isolated in several oral diseases, including pulpal infections, oral abscesses, and periodontitis (11, 26). Cells of anaerobic, gram-negative, and black-pigmented bacteria can be the predominant periodontopathic bacteria in recurrent infections of adult periodontitis. The first pathogenic step involves microbial colonization, and *P. gingivalis* has been shown to adhere to epithelial cells, salivary proteins, and other oral bacteria by using filamentous surface appendages or fimbriae. Although this mechanism is still not completely understood, several observations (28) suggest that the fimbriae or fimbria-like structures play an important role in the adhesion of the bacteria to the tooth or oral epithelial surfaces. Lee et al. (9) have shown that monoclonal antibodies to purified fimbriae and synthetic peptides analogous to the fimbriin sequence block the adherence of *P. gingivalis* to oral epithelial cells and to oral surfaces.

The local virulence factors of *P. gingivalis* do not seem to have a direct effect on bone resorption but stimulate the production of numerous inflammatory cytokines that are able to increase osteoclastic activation. The effect of osteoclastic cells is represented by the damage caused by epithelial attachment, destruction of collagen, and alveolar bone resorption (20). Gingivitis and periodontal diseases also stimulate the local and systemic immune reactions mediated by B cells.

While inflammatory cytokines are thought to be associated with the principal lesions of periodontitis, the humoral immune system might play a role in the mediation of the development of gingivitis and periodontitis, as many investigators have demonstrated (23, 24). The concentrations of immunoglobulins specific to whole bacterial cells, to lipopolysaccharide, and to the fimbrial protein of different periodontopathic bacteria are high in patients with adult periodontitis compared to those in healthy subjects (1, 14, 17, 25). The fimbriae are

really a species-specific component of *P. gingivalis* and are a useful tool for determining human antibody response (27). The specificity of fimbrial antigen is important for detection of a specific immune response because the N-terminal amino acid sequence of fimbriin has been shown to be completely different from those of fimbriins of other bacteria, including *Bacteroides nodosus*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Moraxella nonliquefaciens*, and *Pseudomonas aeruginosa* (28).

The purpose of the present study was to evaluate the prevalence of *P. gingivalis* in subgingival plaque samples and the correlation between culture methods and detection of immunoglobulin A (IgA) specific to *P. gingivalis* fimbrial antigen in gingival crevicular fluid (GCF) of patients with acute recurrent periodontitis. This correlation may lead to the employment of the IgA parameter as a prognostic tool for local *P. gingivalis* activation. Moreover, the titers of humoral IgG specific to the same antigen were also investigated.

MATERIALS AND METHODS

Selection of patients. Thirty-three patients with severe periodontal disease were enrolled from the patients attending the Clinic of Dentistry, Section of Parodontology, of the University of Catania. Periodontal patients were selected if they had severe adult periodontitis with a pocket depth of greater than 5 mm (active site) and at least one inactive site. The patients had no history of periodontal treatment or antibiotic therapy during the previous 6 months. Twenty-one healthy subjects with no periodontal pathology were included in this study as a control group.

Collection of subgingival plaque and microbiological monitoring. Samples were obtained from periodontal pockets after supragingival plaque had been removed from the teeth to be sampled. Forty-six subgingival plaque samples were collected from periodontal patients, in particular, one sample from a single active site from each of 20 patients and one sample from each of two different active sites of the remaining 13 patients. Samples from inactive sites were collected in the same way from the same patients. A single sample was obtained from each of the 21 healthy subjects.

The subgingival plaque samples were inoculated into 2 ml of brucella broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.4- μ l/ml vitamin K₁ (Sigma Chemical Co., St. Louis, Mo.) and 5- μ g/ml hemin (Sigma Chemical Co.). They were then diluted and plated onto Trypticase soy agar (BBL Microbiology Systems) supplemented with 10% defibrinated horse blood (Unipath, Rome, Italy), 5- μ g/ml hemin, and 0.4- μ l/ml vitamin K₁. The plates were incubated in duplicate in an anaerobic atmosphere for 7 to 10 days or in air plus 10% CO₂ for 2 to 4 days. The bacteria grown were selected on the basis of

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size, color, shape, and staining. The anaerobic bacteria were identified by the API 20 and rapid ID 32A (Biomérieux a La Balme, Les Grottes, France) biochemical tests and by gas chromatographic analysis (Perkin-Elmer Instrument) of fatty acids (6). Black-pigmented, anaerobic, gram-negative rods were submitted to a fluorescence test by longwave UV light; absence of fluorescence was considered a rapid taxonomic test to distinguish between *P. gingivalis* and other black-pigmented, anaerobic, gram-negative rods (18).

GCF collection and serum sampling. GCF samples were taken from at least one active and one inactive site from each periodontal patient and from one site of each healthy subject. A filter paper cone (Johnson and Johnson, East Windsor, United Kingdom) was introduced into the gingival crevice until the cone absorbed 0.5 μ l of GCF (3). Cones were stored at -20°C in 100 μ l of phosphate-buffered saline containing 0.05% Tween 20 (elution buffer) until tested. To evaluate the presence of specific antibodies in GCF, the paper cones were eluted by shaking for 30 min at room temperature. A serum sample was obtained from each patient for detection of IgG specific to the *P. gingivalis* fimbrial antigen. Positive and negative serum samples and GCF specimens were selected from our collection by immunofluorescence assay and used as reference specimens.

Fimbrial antigen preparation. Preparation of the fimbrial antigen from *P. gingivalis* 381 was performed by using the modified procedure described by Lee et al. (9).

Protein concentration was evaluated by the method of Lowry et al. (12). The purity of the 43-kDa fimbrial protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with minor modifications of the Laemmli method (7). The crude fimbrial antigen was stored in aliquots at -80°C until used.

IgA and IgG determination in GCF and serum samples. The presence of IgA and IgG antibodies specific to the *P. gingivalis* 381 fimbrial antigen in GCF and serum samples was determined by enzyme-linked immunosorbent microassay.

Polystyrene microtiter strips (Nunc Maxisorp; Nunc, Roskilde, Denmark) were coated with *P. gingivalis* fimbrial antigen in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) by overnight incubation at room temperature. After washing steps, twofold dilutions of each serum sample, from 1:25 to 1:800, in phosphate-buffered saline containing 0.5% bovine serum albumin (Sigma Chemical Co.) and 0.5% Tween 20 (sample buffer) were prepared; GCF specimens were diluted in the sample buffer to a final dilution of 1:200. Diluted GCF samples or dilutions of serum were added to the wells, and the strips were then incubated for 1 h at 37°C . Plates were washed, and peroxidase-conjugated rabbit immunoglobulins to human IgA or IgG (Dako, Glostrup, Denmark) diluted 1:3,000 and 1:15,000, respectively, in sample buffer were added. The plates were then incubated for 1 h at 37°C . After washing steps, 1,2-phenylenediamine in sodium citrate-phosphate buffer (pH 5.0) containing 0.03% hydrogen peroxide was added. Strips were incubated for 30 min at room temperature in the dark, and the reaction was stopped by adding 150 μ l of 1 M HCl per well. Optical density at 492 nm was measured by a Multiskan MCC spectrophotometer (Lab-system, Helsinki, Finland).

Specimens were considered positive if their absorbance values were higher than the mean of negative controls plus 3 standard deviations (cutoff); in the case of a cutoff lower than 0.200, this value was considered to be the new cutoff.

Statistical analysis. Statistical studies were carried out by using the chi-square test.

RESULTS

Microbiological results. *P. gingivalis* was isolated from 36 (78.3%) of 46 samples obtained from active sites of periodontal patients and from 16 (34.7%) of 46 samples from inactive sites of the same patients. *P. gingivalis* was detected in only one specimen (4.7%) from the 21 healthy subjects.

Immunological results. The isolation of *P. gingivalis* was related to the presence of specific IgA in the GCF of the periodontal patients. In particular, 27 (58.7%) of the 46 GCF samples from active sites of periodontal patients were positive for IgA specific to the antigen tested, while only 11 (23.9%) of the 46 inactive sites of the same patients were IgA positive. Of the 46 active sites assayed, 19 (41.3%) were IgA negative, while 35 (76.1%) of the inactive sites were IgA negative.

The overall agreement of both methods for periodontal patients, calculated as the concordance of the isolation results and the IgA detection results, was 65.2% (60 of 92); in particular, the agreement was 71.7% (33 of 46) and 58.6% (27 of 46) for active and inactive sites, respectively. Concerning the discrepant results for both active and inactive sites, 25% (23 of 92) were negative for the detection of specific IgA and positive for the isolation of *P. gingivalis* and 9.7% (9 of 92) were positive for specific IgA and negative for bacterial isolation.

The positive predictive value of the IgA detection assay (27 [71.1%] of 38) was higher than that of the culture method (36 [69.2%] of 52).

No IgA specific to the *P. gingivalis* fimbrial antigen was detected in the GCF of the 21 healthy subjects, and *P. gingivalis* was isolated from only 1 of those subjects.

The statistical analysis carried out to evaluate the relationship between the results obtained by comparing the isolation method and specific IgA antibody detection were significant at $P < 0.01$ for the active sites of both periodontal patients and healthy subjects, while for the inactive sites of periodontal patients, the same relationship was significant at $P < 0.05$.

The titers of IgG antibody to the *P. gingivalis* fimbrial antigen in serum were similar for all of the subjects studied.

DISCUSSION

This study indicates that *P. gingivalis* is one of the major pathogens in the active sites of periodontal patients, since it was isolated from more than 78% of the total sites examined. Different percentages of *P. gingivalis* isolation in recurrent pockets have been reported (3, 4, 19, 20, 29), and these could be due to the different phases of the examination of the bacterial flora. In the early phase of periodontal disease, a high level of *P. gingivalis* presence is detected, while in a later stage, the recurrent pockets may become chronic and they can be colonized by other periodontopathic bacteria, leading to changes in microbial ecology (3).

Many researchers have observed an association between *P. gingivalis* isolation and detection of immunoglobulins, such as specific IgG, especially of the IgG4 subclass, and IgA, in the serum of adults with periodontitis (5, 8, 13, 14, 17, 25).

In this study, the *P. gingivalis* antigen used for IgG and IgA detection was the purified fimbrial antigen instead of the bacterial lysate antigens employed by others (17, 25). The use of the 43-kDa fimbrial antigen should enhance the specificity of the serological method, avoiding the cross-reactions that often occur when bacterial cell extracts are employed in serology. Even though the antigen used in this study was different, our results support those of Schenck et al. (17), indicating no statistically significant differences in the levels of IgG antibodies to *P. gingivalis* detected in serum samples obtained from periodontal patients and healthy subjects. One explanation for this observation could be that since *P. gingivalis* belongs to the normal oral microflora (22), the humoral IgG is often present in both healthy subjects and periodontal patients. We supposed that a better indication of active *P. gingivalis* colonization of the gingival pockets would be a different class of immunoglobulins that can be produced at the site of infection. Many investigators have, in fact, detected antibodies specific to periodontal pathogens in GCF and saliva, suggesting local immunoglobulin production by gingival cells at active periodontal sites (2, 3, 15, 17, 21). Since IgA is one of the earliest immunological responses to infection, especially at a site of infection, our interest was to study the relationship between *P. gingivalis* isolation and a specific IgA response in GCF sampled at sites of periodontal infection.

The analysis of our results indicates that detection of IgA specific to the *P. gingivalis* fimbrial antigen in GCF is a useful tool for the determination of bacterial colonization of gingival pockets. In fact, in the majority of the active-site samples, *P. gingivalis* was isolated and it was significantly correlated with the detection of specific IgA in GCF. Moreover, this correlation is also confirmed because in 80% of the cases in which *P. gingivalis* was not isolated, IgA was not detectable. These results were also supported by those obtained with samples from

inactive sites of the same periodontal patients. At these sites, in fact, both isolation and IgA detection were negative in 76.6% of the cases and in only a few healthy sites was *P. gingivalis* isolated and correlated with the presence of specific IgA. The agreement between the culture method and the serological method, in both active and inactive sites of periodontal patients, confirms the specificity of the IgA method. Since in many cases, one inactive and one active site were sampled from the same tooth, it can be supposed that the bacteria colonize a unique niche of the tooth and the immune response is particularly strong only at the site of colonization.

These results were further confirmed in the group of healthy subjects. In this group, the agreement (95.2%) between the absence of *P. gingivalis* and the absence of a specific local immune response indicates that specific activation of a local IgA response is frequently present in the course of active *P. gingivalis* infection.

As regards the discrepant results obtained for both the active and the inactive sites of periodontal patients, where *P. gingivalis* was isolated in the absence of specific IgA detection, it can be supposed that contamination of the subgingival specimens by the supragingival microflora occurred during sampling because *P. gingivalis* is also usually present in saliva and in the oral microflora (16, 22). On the contrary, the few cases of discrepant results in which IgA was detected in the absence of bacterial isolation could be due to inactivation of *P. gingivalis* during transport of the subgingival plaque samples to the laboratory. Another explanation for this last result could be inability of the culture procedure to detect the target organisms, especially in single subgingival plaque samples taken from periodontally diseased sites (10).

The present study suggests that *P. gingivalis*, which is one of the most important periodontal microorganisms in the early stages of recurrent periodontal disease, is associated with local production of specific IgA. Although the effective role of local IgA in GCF is still unclear, it probably protects gingival tissues against the destructive effects of the periodontopathic bacterial flora, modulating the phase of recurrent periodontal infections.

The presence of specific IgA in these specimens could be a parameter indicating an early phase of periodontal infection. In fact, the immune production of specific IgA in this phase of the disease probably occurs at an early stage of active *P. gingivalis* colonization of the dental pocket.

Studies are still in progress to confirm this hypothesis. If it is confirmed, the detection of specific IgA antibodies in GCF by enzyme-linked immunosorbent assay may be useful as a predictive parameter to reveal the early phase of the activation of recurrent periodontal infection and to suggest to the periodontist the type and frequency of specific antibacterial periodontal treatment. Therapy could be more effective if it were applied in the early phase of periodontal disease.

The easy sampling of GCF, the absence of any contamination of the sample by interfering factors, the simple way of detecting IgA compared with culture methods, and the good correlation between specific IgA detection and *P. gingivalis* isolation make this immunological method an appropriate diagnostic tool for the determination of recurrent periodontal disease.

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REFERENCES

- Brandtzaeg, P., I. Fjellanger, and S. T. Gjeruldsen. 1970. Human secretory immunoglobulins. Salivary secretions from individuals with normal or low levels of immunoglobulins. *Scand. J. Haematol.* **12**:1-83.
- Byers, C. W., P. D. Toto, and A. W. Garguilo. 1975. Levels of immunoglobulins IgG, IgA, and IgM in the human inflamed gingiva. *J. Periodontol.* **48**:387-390.
- Choi, J. I., T. Nakagawa, S. Yamada, I. Takazoe, and K. Okuda. 1990. Clinical, microbiological and immunological studies on recurrent periodontal disease. *J. Clin. Periodontol.* **17**:419-425.
- Dahlen, G., G. Manji, V. Baelum, and O. Fejrskov. 1992. Putative periodontopathogens in "diseased" and "non-diseased" persons exhibiting poor oral hygiene. *J. Clin. Periodontol.* **19**:35-42.
- De Nardin, A. M., H. T. Sojar, S. G. Grossi, L. A. Christersson, and R. J. Genco. 1991. Humoral immunity of older adults with periodontal disease to *Porphyromonas gingivalis*. *Infect. Immun.* **59**:4363-4370.
- Holdeman, L. V., and W. E. C. Moore (ed). 1972. Anaerobe laboratory manual, 2nd ed. Virginia Polytechnic Institute and State University, Blacksburg.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.
- Lamster, I. B., R. Celenti, and J. L. Ebersole. 1990. The relationship of serum IgG antibody titers to periodontal pathogens to indicators of the host response in crevicular fluid. *J. Clin. Periodontol.* **17**:419-425.
- Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco. 1992. Synthetic peptides analogous to the fimbriin sequence inhibit adherence of *Porphyromonas gingivalis*. *Infect. Immun.* **60**:1662-1670.
- Loesche, W. J., D. E. Lopatin, J. Stoll, N. van Poperin, and P. P. Hujuel. 1992. Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? *J. Clin. Microbiol.* **30**:418-426.
- Loos, B. G., A. J. Van Winkeloff, R. G. Dunford, R. J. Genco, J. De Graaff, D. P. Dickinson, and D. W. Dyer. 1992. A statistical approach to the ecology of *Porphyromonas gingivalis*. *J. Dent. Res.* **71**:353-358.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Magnusson, I., R. G. Marks, W. B. Clark, C. B. Walker, S. B. Low, and W. P. McArthur. 1991. Clinical, microbiological and immunological characteristics of subjects with "refractory" periodontal disease. *J. Clin. Periodontol.* **18**: 291-299.
- Markkanen, H., S. M. Syrjanen, and P. Alakuijala. 1986. Salivary IgA, lysozyme and β 2-microglobulin in periodontal disease. *Scand. J. Dent. Res.* **94**:115-120.
- Marttala, W. H., P. D. Toto, and A. W. Garguilo. 1974. Identification of fluorescent antibodies in periodontitis. *J. Periodontol.* **45**:853-861.
- Petit, M. D. A., T. J. M. van Steenberg, M. F. Timmerman, J. de Graaff, and U. van der Velden. 1994. Prevalence of periodontitis and suspected periodontal pathogens in families of adult periodontitis patients. *J. Clin. Periodontol.* **21**:76-85.
- Schenck, K., D. Poppelsdorf, C. Denis, and T. Tollefsen. 1993. Levels of salivary IgA antibodies reactive with bacteria from dental plaque are associated with susceptibility to experimental gingivitis. *J. Clin. Periodontol.* **20**:411-417.
- Slots, J., and H. S. Reynolds. 1982. Long-wave UV light fluorescence for identification of black-pigmented *Bacteroides* spp. *J. Clin. Microbiol.* **16**: 1148-1151.
- Tanner, A. C. R., C. Haffer, G. T. Bratthal, R. A. Visconti, and S. S. Socransky. 1979. A study of the bacteria associated with advancing periodontitis in man. *J. Clin. Periodontol.* **6**:278-307.
- Tanner, A. C. R., S. S. Socransky, and J. M. Goodson. 1984. Microbiota of periodontal pockets losing crestal alveolar bone. *J. Periodontol. Res.* **19**: 279-291.
- Tew, J. G., D. R. Marshall, J. A. Burmeister, and R. R. Ranney. 1985. Relationship between gingival crevicular fluid and serum antibody titers in young adults with generalized and localized periodontitis. *Infect. Immun.* **49**:487-493.
- Van Steenberg, T. J. M., M. D. A. Petit, L. H. M. Scholte, U. van der Velden, and J. de Graaff. 1993. Transmission of *Porphyromonas gingivalis* between spouses. *J. Clin. Periodontol.* **20**:340-345.
- Wilton, J. M. A., J. L. M. Bampton, C. S. Griffiths, M. A. Curtis, J. S. Life, N. W. Johnson, J. R. Powell, G. J. Harrap, and P. Critchley. 1992. Interleukin-1 beta (IL-1 β) levels in gingival crevicular fluid from adults with previous evidence of destructive periodontitis. *J. Clin. Periodontol.* **19**:53-57.
- Wilton, J. M. A., M. A. Curtis, I. R. Gillett, G. S. Griffiths, M. F. J. Maiden, J. A. C. Sterne, D. T. Wilson, and N. W. Johnson. 1989. Detection of high risk groups and individuals for periodontal diseases: laboratory markers from analysis of saliva. *J. Clin. Periodontol.* **16**:475-483.
- Wilton, J. M. A., T. J. Hurst, and A. H. Austin. 1992. IgG subclass antibodies to *Porphyromonas gingivalis* in patients with destructive periodontal disease. *J. Clin. Periodontol.* **19**:646-651.
- Wolff, L. F., D. M. Aeppli, B. Pihlstrom, L. Anderson, J. Stoltenberg, J. Osborn, N. Hardie, C. Shelburne, and G. Fisher. 1993. Natural distribution

- of 5 bacteria associated with periodontal disease. J. Clin. Periodontol. **20**: 699-706.
27. Yoshimura, F., K. Takahashi, Y. Nodasaka, and T. Suzuki. 1984. Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. J. Bacteriol. **160**:949-957.
28. Yoshimura, F., T. Takasawa, M. Yoneyama, T. Yamaguchi, H. Shiokawa, and T. Suzuki. 1985. Fimbriae from the oral anaerobe *Bacteroides gingivalis*: physical, chemical, and immunological properties. J. Bacteriol. **163**:730-734.
29. Zimmer, W., M. Wilson, P. D. Marsh, H. N. Newman, and J. Bulman. 1991. *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in the plaque of children without periodontitis. Microb. Ecol. Health Dis. **4**:329-336.