Colocalization of Porphyromonas gingivalis with CD4+ T cells in periodontal disease

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Abstract
Porphyromonas gingivalis, an anaerobic, asaccharolytic gram-negative bacterium, is a causative agent in chronic periodontitis. It has many virulence factors that facilitate infection of the gingiva, but little is known about the local immune cells that respond to this bacterium. The aims of this study were to quantify P. gingivalis in gingival biopsies from patients with periodontitis using laser capture microdissection (LCM) plus qRT-PCR and to determine the phenotype of immune cells associated with the bacteria using immunofluorescence. The presence of P. gingivalis was confirmed in periodontitis gingival tissue from 10 patients, and differences in bacterial distribution in the epithelium and connective tissue with or without inflammatory infiltrates were observed. Immune cells found in the biopsy tissues, including CD20+ mature B cells and CD138+ plasma cells, were associated with the Th2-type immune response. Most P. gingivalis was in direct contact with CD4+ T cells. This study revealed for the first time the colocalization of P. gingivalis with immune cells. Use of LCM combined with qRT-PCR enabled quantitative analysis of bacteria in a selected area of a biopsy sample without any tissue degradation. Observation of the immune cells associated with these bacteria was also performed by immunofluorescence.

Introduction
Periodontal disease is initiated by the accumulation of specific anaerobic bacteria in the gingival sulcus and involves a complex interaction of the bacteria with host immune cells (Papapanou et al., 2009). This presumably represents a challenge to the host in terms of maintaining immune homeostasis, yet little is known about the subset of immune cells that respond to this flora (Teng, 2006a, b; Kim et al., 2010). Specific pathogens within the plaque biofilm, such as Porphyromonas gingivalis, induce a strong humoral immune response during periodontitis (Califano et al., 1999).

Porphyromonas gingivalis, a gram-negative oral anaerobe, is strongly associated with adult periodontitis (Cutler et al., 1995). Specifically, the bacterium is a component of subgingival plaque that interfaces with gingival tissue. Because of its many virulence factors, such as proteases, P. gingivalis can modulate host cytokine signaling networks and generate inflammatory infiltrates that are responsible for the chronic nature of periodontitis. Previous studies have shown that P. gingivalis can survive, spread to neighboring host epithelial cells, and resist phagocytosis in vitro (Cutler et al., 1993; Miyabe et al., 2004). In vivo, P. gingivalis has been identified in pathological gingiva using several methods, including immunofluorescence, immunohistochemistry, and fluorescence in situ hybridization (Rudney et al., 2005; Kim et al., 2010).

In the present study, we examined biopsy samples from patients with periodontitis to gain insights into the interactions of host immune cells and P. gingivalis in periodontal disease. The aims were to detect P. gingivalis in biopsy samples and to determine the phenotype of the immune cells associated with these bacteria. Toward this end, we used laser capture microdissection (LCM) to extract RNA from samples followed by the quantification...
of bacteria using qRT-PCR. In parallel, we performed immunofluorescence experiments to study the distribution of immune cells associated with *P. gingivalis* in gingival biopsies from periodontal sites.

**Materials and methods**

**Gingival tissue and section preparation**

Gingival biopsies were obtained from 10 patients who underwent dental surgery for periodontal disease. Oral informed consent was obtained from each patient. Before surgery, the depth of the periodontal pocket was noted, and a subgingival plaque sample was taken with a paper point. Serial 7-μm sections of pathologic gingival tissue were cut on a standard cryotome at −20 °C (Leica, Milton Keynes, UK) and stained using a HistoGene microdissection kit (Molecular Devices, Arcturus, Sunnyvale, CA).

**Detection of *P. gingivalis* in subgingival plaque samples**

The paper points were incubated in water at 37 °C with shaking for 24 h. After the paper points were removed, the DNA was amplified by PCR (GoTaq Polymerase; Promega, Madison, WI) with primers specific for the 16S RNA gene. The sequences were 5′-TGGGTTTAAAGGGTGCGTAG-3′ for the forward primer and 5′-CAATCGAGTTCCTCGTGAT-3′ for the reverse primer (Meuric et al., 2008).

**Laser capture microdissection**

After HistoGene staining, sections were microdissected using a Veritas LCM system (Molecular Devices). For each sample, approximately 1 mm² of each site of interest was microdissected with a separated ‘cap’ (Capsure Macro LCM Caps; Molecular Devices, Arcturus). Three main gingival tissue structures were microdissected (Fig. 1): epithelium, connective tissue without infiltrates, and inflammatory infiltrates in connective tissue.

**RNA extraction**

RNA was extracted from microdissected sections with the PicoPure RNA isolation kit (Molecular Devices) according to the manufacturer’s protocol. Total RNA extract was eluted in 30 μL of water. RNA from cultured *P. gingivalis* (ATCC 33277) was extracted with the RNAeasy mini kit (Qiagen, Hilden, Germany) and used as a positive control. For all samples, the quantity and quality of RNA were measured with the Nanodrop 1000 (Nanodrop, Wilmington, DE).

**qRT-PCR**

Reverse transcription (RT) was performed using M-MLV transcriptase (Promega, Madison, WI) according to the manufacturer’s protocol. For each microdissected sample, an RT reaction without reverse transcriptase was performed to check for the presence of genomic DNA. Primers from Meuric et al. (Meuric et al., 2008) were used to detect *P. gingivalis* 16S RNA. Quantitative PCR was performed using the qPCR Master Mix Plus for Sybr Green I (Eurogentec, Liege, Belgium), 2 μL of cDNA, and 0.4 μM primer. For each sample, measurements of the 16S RNA gene were taken in triplicate. Threshold cycle (Ct) values were converted into number of bacteria (normalized for 1 ng of total RNA) by comparison with a standard curve constructed using serial dilutions of cDNA from *P. gingivalis* 33277. A *P* value was determined to compare epithelium and connective tissue with or without inflammatory infiltrates in each biopsy sample using the analysis of variance (ANOVA) test (EZANOVA software).

![Fig. 1. Seven-micrometer sections of gingival tissue affected by chronic periodontitis were analyzed with the Histogene Microdissection Kit before (left) and after (right) LCM capture. Three regions were analyzed: (a) epithelium (purple), which had a typical organized structure; (b) inflammatory infiltrates, which had high cellular density and no specific organized structure; and (c) connective tissue, which had low cellular density.](image-url)
**Immunofluorescence microscopy**

Monoclonal mouse antibodies against human CD3, CD138, CD14, CD5, CD27, CD4, and CD8 were obtained from Beckman Coulter (Villepinte, France), and goat anti-CD20 antibody was obtained from Neomarkers (Fremont, CA). Rabbit anti-*P. gingivalis* ATCC 33277 was produced in our laboratory by injection of *P. gingivalis* 33277 whole-cell extract. Secondary antibodies used for this study were fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat or anti-rabbit antibody (Jackson Immuno-Research, West Grove, PA) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey antimouse or anti-goat antibody (Jackson ImmunoResearch). Mouse IgG, rabbit IgG (Jackson ImmunoResearch), or goat IgG (Rockland, Gilbertsville, Pennsylvania) were used for negative controls.

Sequential 4-mm-thick cryostat sections were cut and mounted onto poly-L-lysine-coated slides. The slides were incubated for 40 min at room temperature with the appropriate mouse mAb (anti-CD5, anti-CD138, anti-CD14, anti-CD27, anti-CD4, or anti-CD8) along with goat anti-CD20 antibody. After three washes in phosphate-buffered saline (PBS), the slides were incubated for another 40 min with FITC-conjugated donkey anti-mouse antibody along with TRITC-conjugated donkey anti-goat antibody in PBS supplemented with 2% donkey serum (Sigma, St. Louis, MO). After five rinses, the sections were fixed with 4% cold paraformaldehyde and analyzed with the TCS-NT Leica confocal imaging system (Leica Microsystems, Wetzlar, Germany). Neither of the negative controls [mouse anti-IgG (Jackson ImmunoResearch) plus FITC-conjugated donkey anti-mouse antibody or goat anti-IgG plus TRITC-conjugated donkey anti-goat antibody] showed background fluorescence.

**Results**

**Association of *P. gingivalis* in subgingival samples with *P. gingivalis* cell number in biopsy samples**

Ten subgingival samples collected on paper points were assayed for the presence of *P. gingivalis* using standard PCR to amplify the *P. gingivalis* 16S RNA gene. In parallel, the 10 corresponding biopsies were analyzed by qRT-PCR after LCM. PCR detection of *P. gingivalis* in the subgingival samples and the analysis of microdissected tissue by qRT-PCR in the 10 corresponding biopsies are summarized in Fig. 2, which also shows the depth values of the corresponding periodontal pockets.

The two methods used for *P. gingivalis* detection yielded concordant results. That is, the presence of bacteria in gingival tissue was confirmed in all biopsies that corresponded to positive subgingival samples. However, in terms of the quantity of *P. gingivalis* found in the tissue and the pocket depth, deeper pockets did not always correspond to more bacteria.

The amount of bacteria varied among the biopsies and in the different regions of the tissue. In four biopsies, *P. gingivalis* was predominant in a single tissue structure, i.e. it was mainly in either the epithelium or the inflammatory infiltrates.

**Identification of immune cell populations in inflammatory infiltrates of gingival biopsy specimens**

To investigate the immune response to *P. gingivalis* infection, biopsies were stained using antibodies against cell surface molecules that distinguish immune cell populations (CD markers) and examined using immunofluorescence microscopy. The sample containing the most *P. gingivalis* was analyzed (sample 4). The antibodies used in this study allowed us to study and distinguish between the innate and acquired immune responses (Table 1).

The cells involved in the immune response were identified using an anti-CD20 antibody, which specifically binds to mature B cells, in combination with antibodies to cell surface markers typical of T cells (CD3), macrophages (CD14), or plasma cells (CD138) (Fig. 3). Macrophages were the least abundant immune cells, and plasma cells were the most frequently observed immune cells along with CD20+ B cells. Staining with the anti-CD3 antibody revealed the presence of T cells as well.

We next performed a detailed analysis of the B- and T-cell lineages involved in the immune response using antibodies specific for different B-cell (CD5, CD24, and CD27; Fig. 4) and T-cell (CD4 and CD8; Fig. 5) lineages. CD20+ B cells were surrounded by CD138+ plasma cells (Fig. 3). An overlay of green-staining CD20 with red-staining CD5 (Fig. 4) established that only a few CD20+ B cells expressed CD5 in the gingival biopsy specimens. Some of the B cells expressed CD27+ (yellow staining), suggesting that they might be memory B cells. No naïve transitional B cells (CD24−) were observed (Fig. 4). The phenotype of substantial numbers of B cells confirmed the chronic nature of the periodontitis infection.

Regarding T cells, CD4+ T cells were often found adjacent to CD20+ B cells (Fig. 5). Cytotoxic CD8+ T cells were also present but were less abundant. Inflammatory infiltrates mostly comprised a mix of CD3+ CD4+ T cells along with mature B cells (CD20+) and plasma cells (CD138+).
The presence of CD4+ T cells and *P. gingivalis* in biopsies

*Porphyromonas gingivalis* was observed in the biopsies by immunofluorescence microscopy using a polyclonal antibody against *P. gingivalis* to analyze the same sample used for the identification of immune cell populations. After LCM analysis, immunofluorescence confirmed the presence of *P. gingivalis*. We also found that *P. gingivalis* was associated with immune cells, especially with CD4+ T cells. The immunofluorescence images showed clearly that *P. gingivalis* localized preferentially with CD4+ T cells and with CD20+ B cells, but not with CD8+ T cells (Fig. 6).
Porphyromonas gingivalis quantification in gingival biopsy

In this preliminary study, which used a novel combination of techniques to detect *P. gingivalis* in 10 patients, we observed concordant results regarding the presence of *P. gingivalis* in subgingival samples and in gingival biopsies. Concerning pocket depth and *P. gingivalis* invasion, Thiha *et al.* suggested that an elevated load of tissue-invading bacteria seemed to be associated with a tissue-destructive form of periodontitis (Thiha *et al.*, 2007). In contrast, our study suggested that an advanced stage of periodontitis does not always correspond to high levels of bacteria in gingival tissue.

Only a few studies have detected *P. gingivalis* in tissues. Kim *et al.* (2010) used digoxigenin-labeled DNA probes for *in situ* hybridization to detect *P. gingivalis* in tissues. This technique detected infectious microorganisms in tissues and provided some histological information. However, the levels of *P. gingivalis* in the biopsies must be high to be detected with this technique owing to its low sensitivity (Kim *et al.*, 2010). In addition, this method has a major disadvantage in that it uses enzyme digestion, which damages the tissue, especially the epithelium. In contrast, the LCM technique used here allows tissue to be preserved for histological examination, and the same tissue can be used for qRT-PCR and histological observations. Moreover, LCM combined with qRT-PCR enables the identification of bacterial virulence factors in the tissue. However, it would be necessary to improve this technique to increase levels of bacterial RNA from biopsy (Klitgaard *et al.*, 2005).

Our results for biopsy 1 and biopsy 2 (Fig. 2.) were in agreement with previous studies, which found that *P. gingivalis* was located mainly in epithelial tissue (Pekovic & Fillery, 1984; Vitkov *et al.*, 2005; Colombo *et al.*, 2007). The epithelium is the portion of gingival tissue that is in contact with periodontopathogenic bacteria. Vitkov *et al.* (2010) showed that bacterial adhesion to epithelial cells could trigger colonization of gingival tissue and even

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Discussion

*Porphyromonas gingivalis* quantification in gingival biopsy

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| Fig. 3. Immunostaining of immune cells in gingival biopsy 4. Immunohistochemical staining was performed using an anti-CD20 antibody and an FITC-conjugated secondary antibody (green) as well as CD3/CD138/CD14 antibodies bound to TRITC-conjugated secondary antibodies. |
restrict the formation of bacterial biofilms (Vitkov et al., 2005). The present study confirmed the presence of P. gingivalis in epithelium. Internalization of P. gingivalis by epithelial cells was observed previously in cultured cells infected in vitro, and our results suggest that bacteria are similarly internalized in vivo (Duncan et al., 1993; Sandros et al., 1994; Lamont et al., 1995; Njoroge et al., 1997).

**Phenotype of immune cells in inflammatory infiltrates**

After using LCM and qRT-PCR to detect P. gingivalis in biopsies, immunohistochemistry was used to determine the types of immune cells in the inflammatory infiltrates to determine the type of immune response elicited by P. gingivalis.
Moskow and Polson reported in 1991 that in a collection of 350 autopsy and surgically retrieved jaw sections, the types of inflammatory cells in inflamed gingiva and the distribution patterns of the cells varied greatly from individual to individual (Moskow & Polson, 1991). However, our gingival biopsies were all obtained from patients who underwent dental extraction for advanced (terminal) periodontal disease, which is associated with a predominance of plasma cells (Page & Schroeder, 1976). Indeed, use of immunofluorescence to observe different CD markers showed that B cells were the most abundant immune cells in inflammatory infiltrates. Only a few macrophages (CD14+) were found in the lesions; thus, we focused mainly on the immune adaptive response. It seemed likely that it was a Th2 response (Jotwani et al., 2001; Berglundh & Donati, 2005), so most of the CD antibodies used were specific to B cells.

Several investigators have attempted to elucidate the Th1/Th2 profile in periodontal disease. However, the results have generally been difficult to interpret because of differences in the materials examined and the methods used. Immune cells have been studied in tissue in situ, in cells extracted from gingival tissue, in peripheral blood mononuclear cells, in T cell lines and clones, and in purified cell populations. A variety of techniques have been used, including flow cytometry, enzyme-linked immunosorbent assay (ELISA), in situ hybridization, and RT-PCR. In addition, bacterial components, including sonicated bacteria, heat- and formalin-killed cells, outer membrane components, and purified antigens have all been used to stimulate cells in vitro. These different methods could give discordant results, making comparison of cytokine profiles difficult (Zadeh et al., 1999; Manakil et al., 2001; Nakajima et al., 2005; Bodet et al., 2006). LCM and qRT-PCR allow a more precise analysis of cytokine production and bacterial profiles in tissue in vivo and may be useful for investigating the causes of multifactorial periodontal disease.

The predominance of plasma cells in periodontitis is well established (Berglundh & Donati, 2005; Berglundh et al., 2007) and was confirmed by the present study. B cells were present in the inflammatory infiltrates but were differentiated, for the most part, into plasma cells. This could be due to changes in the cytokine environment. However, the relative predominance of B cells and plasma cells in periodontic lesions cannot be explained by enhanced Th2 function alone; there must also be an imbalance between Th1 and Th2. Autoimmune reactions are evident in periodontitis lesions (Ali et al., 2011). The role of autoantibodies in the regulation of host response in periodontitis, however, needs to be clarified. This process could be investigated in detail by qRT-PCR analysis of samples.

Fig. 6. Immunostaining of immune cells and Porphyromonas gingivalis in gingival biopsy 4. Immunohistochemical staining was performed using a P. gingivalis antibody and an FITC-conjugated secondary antibody (green) and CD4, CD8, or CD20 antibodies and TRITC-conjugated secondary antibodies (red). All images except the last were acquired with a 63 × objective lens. A 100 × objective was used for the last image.
Colocalization of *P. gingivalis* with immune cells in gingival biopsies

Double staining of *P. gingivalis* and different immune cell populations showed the association of CD4+ T cells with *P. gingivalis*, indicating that these immune cells may be recruited to the infection sites. Previous studies proved the existence of a CD4+ T-cell-rich area in the lamina propria in periodontal gingival biopsies and suggested that these cells may be involved in the chronicity of the disease (Takeichi et al., 2000; Yamazaki et al., 2000; Jotwani et al., 2001). CD4+ T cells can modulate cytokine production in gingival tissue and generate a destructive (Th2) or protective (Th1) immune response. Thus, *P. gingivalis* could modulate the immune response and contribute to the inflammation of the tissue. The presence of *P. gingivalis* in inflammatory infiltrates was interesting and provided evidence that there were interactions between these bacteria and immune cells. Previous studies showed that *P. gingivalis* can survive in host cells such as gingival epithelial cells (Yilmaz, 2008). However, this is the first time that colocalization of *P. gingivalis* with CD4+ T cells was observed in ‘ex vivo’ samples. The infection mechanism of T cells by *P. gingivalis* remains unknown and could be a new direction of study in the effort to understand periodontitis.

Conclusion and perspectives

To the best of our knowledge, this study is the first to show that *P. gingivalis* colocalized with immune cells using two different methods (immunofluorescence and LCM plus qRT-PCR). Specifically, investigation into biopsies from patients with advanced-stage periodontitis revealed that *P. gingivalis* was in contact with immune cells: the bacteria were adjacent to CD4+ T cells and CD20+ B cells, confirming a Th2-type immune response to the invasion by periodontal bacteria.

The results of this preliminary study need to be confirmed with more patients. Furthermore, the expression of genes involved in the immune response could be analyzed in immune cells that are closely associated with the bacteria. Host protein citrullination by *P. gingivalis* peptidylarginine deiminase could be analyzed using anticitrulline antibodies to study the link between rheumatoid arthritis, autoimmune disease, and periodontal disease (Detert et al., 2010; Wegner et al., 2010).

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