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Periodontology

Intracellular localization of *Porphyromonas gingivalis* thiol proteinase in periodontal tissues of chronic periodontitis patients

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OBJECTIVES: ***Porphyromonas gingivalis*** **is a significant periodontal pathogen that has been shown** ***in vitro*** **to be able to invade gingival epithelial cells and grow intracellularly. The aim of the present study was to detect** ***P. gingivalis*** **in gingival tissues from chronic periodontitis (CP) patients.**

MATERIALS AND METHODS: Monoclonal antibodies specific to a cell membrane-bound thiol proteinase of ***P. gingivalis*** **were used to detect the microbe in gingival tissues of CP patients (*n* = 13) by immunohistochemistry. The presence of** ***P. gingivalis*** **was also analysed by polymerase chain reaction (PCR).**

RESULTS: Immunohistochemical analysis of the periodontal tissues revealed positive staining for ***P. gingivalis*** **thiol proteinase in 11 of the 13 patients. Positive staining was mainly located intracellularly in the perinuclear region of the cytoplasm in the periodontal epithelial cells and it could be detected throughout the whole depth of both pocket and oral epithelium. The sensitivity of immunohistochemistry was found to be comparable with that of PCR.**

CONCLUSIONS: Our results provide ***in vivo*** **evidence of the ability of** ***P. gingivalis*** **to enter human gingival epithelial cells. Intracellular localization of** ***P. gingivalis*** **contributes to its evasion of the host immune surveillance and eventually increases its resistance to conventional treatments of periodontal diseases.**

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Keywords: *Porphyromonas gingivalis*; cellular invasion; immune evasion; chronic periodontitis; thiol proteinase

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Introduction

Periodontal disease is a general designation for the inflammatory host response of the gingiva and underlying connective tissue to bacterial accumulations on the teeth and involves irreversible destruction of the soft and hard tooth supporting structures (Hamada *et al*, 1991; Haffajee and Socransky, 1994). The excessive production and uncontrolled activation of the proinflammatory mediators, particularly IL-1, TNF-α, prostaglandins, complement and host derived matrix metalloproteinases (MMPs) lead to the tissue damage of periodontitis (Rautemaa and Meri, 1996; Tervahartiala *et al*, 2000).

The majority of putative periodontal pathogens in chronic periodontitis (CP) are gram-negative anaerobic rods. *Porphyromonas gingivalis* has been strongly implicated in the aetiology of CP (Lamont and Jenkinson, 2000). It is a gram-negative, anaerobic, non-motile, short rod of the black-pigmented *Bacteroides* group, the members of which produce an exceptionally large array of virulence factors like many proteases, endotoxin, NH_3 and H_2S (Kadowaki *et al*, 2000). In response to an infection by *Bacteroides*-bacteria a local and systemic immune response develops. In general, *P. gingivalis* is one of the most important pathogens in advanced periodontitis. It is known to be proteolytically highly active, and the proteases have been found to have specific roles in its virulence (Schenkein and Berry, 1988; Sorsa *et al*, 1992; DeCarlo *et al*, 1999; Yun *et al*, 1999). *Porphyromonas gingivalis* elaborates a number of extracellular and cell-bound proteases that can directly degrade the host periodontal tissues (Birkedal-Hansen *et al*, 1993). It also enhances the expression and activation of the host cell derived MMPs (Hamada *et al*, 1992; Ding *et al*, 1997; DeCarlo *et al*, 1998). In addition, it has proteases

that can inactivate host's immune system, especially the complement system and thus downregulate the inflammatory response (Schenkein, 1989). Some *P. gingivalis* proteases can modify host cell surfaces to expose cryptotopes.

Intracellular localization of any microbe is always regarded as an important immune evasion mechanism and a virulence factor (Rautemaa and Meri, 1999) and *P. gingivalis* has thus been given much attention (Dorn *et al*, 2002). It has been demonstrated *in vitro* that *P. gingivalis* can attach to and penetrate into gingival epithelial cells as well as endothelial cell lines (Lamont *et al*, 1992; Sandros *et al*, 1993, 1994). Recently, intracellular localization has also been demonstrated on buccal epithelial cells both *in vitro* and *in vivo* (Rudney *et al*, 2001). *P. gingivalis* fimbria have been found to be important in host cell adherence as their binding stimulates cell surface-associated adhesion molecules in cell culture (Khlgatian *et al*, 2002; Nakagawa *et al*, 2002). Using specific monoclonal antibodies against a *P. gingivalis* outer membrane protein, a thiol metalloproteinase, we here demonstrate for the first time intracellular localization of *P. gingivalis* in gingival tissues *in vivo*.

Materials and methods

Samples

Surgical samples of diseased gingiva (CP) were obtained from patients with moderate to severe generalized adult type CP ($n = 13$; eight women and five men; 52.2 ± 10.9 years) as judged by clinical measurements of pocket depths, loss of attachment, radiographic bone loss, suppuration and gingival bleeding on probing. The patients had radiographic evidence for bone loss of 20–50% on many teeth and a mean loss of attachment ranging from 4 to 6 mm. Samples were obtained from premolar-molar regions during flap surgery of the initial periodontal therapy. The patients did not receive any antimicrobial therapy or professional periodontal treatment to the sampling area prior to operation. Control tissue specimens ($n = 15$; two samples of healthy gingiva, one keratocyst, one follicular cyst, seven periapical granulomas, one sample of pancreas, two samples of *Helicobacter pylori*-infected gastric corpus and one sample of non-infected gastric corpus) were randomly selected from the files of the Department of Pathology, University of Helsinki. All specimens were formalin-fixed and paraffin-embedded. The study was approved by the Ethical Committee of the Institutes of Dentistry, University of Helsinki and the subjects were enrolled into the study and treated in compliance with the Helsinki Agreement as revised in 1983.

Bacterial culture

Subgingival plaque samples were collected from deep suppurating periodontal pockets (> 5 mm) of all CP patients prior to flap surgery operations and antimicrobial therapy. The samples were transported in VMGA III medium (viability-maintaining Gothenburg anaerobe medium) (Möller, 1966) and 10-fold dilutions (10^0–10^{-5}) were cultured routinely on brucella blood agar plates (brucella agar medium; BBL, Cockeysville, MD, USA; supplemented with 5% defibrinated horse blood, 5 mg hemin ml^{-1}, and 10 mg vitamin K ml^{-1}) at the Oral Microbiology Unit of the Helsinki University Central Hospital Laboratory Diagnostics. After 7 days of anaerobic incubation (80% N_2, 10% H_2, 10% CO_2) the *P. gingivalis*-bacteria were identified based on the colony morphology and pigmentation, staining and biochemical reactions (Jousimies-Somer *et al*, 2003).

Antibodies

Mouse monoclonal antibodies (designated VA_1 and IIB_2) against *P. gingivalis* thiol proteinase were used as primary antibodies (DeCarlo, 1994, 1997, 1999). To prepare the antibodies *P. gingivalis* ATCC 33 277^T grown anaerobically at 37°C on blood agar plates were transferred to enriched anaerobic glucose broth (Syed and Loesch, 1978) and then incubated for 10 days at 37° C in an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. At the end of the incubation, cells and debris were removed by passage through a 0.2 μm filter and the filtrate was then concentrated 20-fold by ultra-filtration using a 10 000 molecular mass cut-off filter in a Millipore Pellicon ultra-filtration device (Millipore Corp., Bedford, MA, USA.). During concentration the medium was exchanged with 50 mM Tris-HCl buffer, pH 8.1, 0.01 M NaCl. The concentrate was passed over an Arginine-Sepharose column (Arginine-Sepharose 4-B, Pharmacia, Uppsala, Sweden) at 4°C. 1 M L-Arginine (monohydrochloride, Sigma, St Louis, MO) in 50 mM Tris-HCl, pH 7.5, 0.001 M NaCl was used as the elution buffer after extensive washes. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as standard. After partial purification by gelatin-Sepharose chromatography the proteins in the *P. gingivalis* culture broth were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis under non-denaturing conditions and visualized by staining with $CuCl_2$ (Lee *et al*, 1987). A highly proteolytic band in the region of 100–110 kDa, identified by zymography (copolymerization, overlay and back-diffusion zymography), was excised and pulverized by passage through a 20 gauge needle.

The monoclonal antibodies were made essentially by the method of Kearney (Kearney, 1984) as previously described (Birkedal-Hansen *et al*, 1988). The arginine-Sepharose eluate was dissociated by precipitation in 20 volumes of 0.05% TFA in acetonitrile then resolublized in phosphate-buffered saline (PBS) containing 50 mM dithiothreitol (DTT) by heating to 100°C for 10 min. Balb/C mice were immunized over a 3-week period by biweekly injections using 50 μg of the denaturated arginine-Sepharose eluate into the foot pads and inguinal regions. Spleen and regional lymph nodes were excised, and cells were collected by teasing and fused with murine myeloma cells PX63-Ag8.653. Hybridomas were selected in HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine; ICN Biomedicals, Costa Mesa, CA, USA) medium and

screened against the denaturated Arginine-Sepharose eluate by enzyme-linked immunosorbent assay and Western analysis. Useful hybridomas were cloned and recloned by limiting dilution. Ascites were produced by intraperitoneal injection of pristane primed Balb/C mice with 1×10^6 hybridoma cells suspended in PBS. Ascites fluid was collected by aspiration and immunoglobulin (Ig) was purified by passage over a Protein-A Sepharose affinity column following a 10-fold dilution with 50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The column was washed with the same antibody and the bound antibody was eluted with 0.1 M acetic acid, 0.2 M NaCl, pH 3, and immediately neutralized with Tris-HCl, pH 7.5. Two mAbs (VA_1 and IIB_2) were used in this study. The specificity of the antibodies for the highly proteolytic *P. gingivalis* antigen was tested by immunoblotting (DeCarlo, 1994).

Immunohistochemical staining

Six consecutive, 4-μm thick, formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol series and in water. Sections were incubated in pepsin (5 mg pepsin, 5 ml H_2O, 50 μl 1-N HCl) for 45 min in a humid chamber and washed three times for 5 min in PBS buffer. In some instances bovine serum albumin (BSA; Behringverke Gmbh, Marburg, Germany) was added to PBS (PBS-BSA) to reduce non-specific reactions. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 in methanol for 30 min, then the sections were washed with PBS three times for 5 min. A modification of the Vectastain® (Vector Laboratories, Burlingame, CA, USA) Kit protocol was used. The sections were first incubated in normal goat blocking serum from the Kit diluted 1:50 in 2% PBS-BSA. The sections were then incubated with the primary antibodies against *P. gingivalis* bacteria (5 μg ml^{-1} diluted in 1% PBS-BSA) for 30 min at 37°C and then kept overnight at 4°C in a humid chamber. Control stainings were performed by omitting the primary antibody. The next day after been washed three times the sections were incubated for 30 min at 37°C with biotinylated anti-mouse IgG secondary antibody solution from the Kit diluted 1:250 into 0.1% PBS-BSA. After three washes the sections were incubated with the Kit reagent for 30 min at 37°C and then washed three times with buffer. Peroxidase binding sites were revealed with 3-amino-9-ethylcarbazole (AEC; 0.05 M acetic acid, 0.05 M sodium acetate, pH 5.0; 3-amino-9-ethylcarbazole, N,N-dimethylformamide) with 0.03% hydrogen peroxidase. Finally, the slides were washed with tap water and then counter stained with Mayer's haematoxylin for 4 min and again rinsed with tap water before mounting with glycergel (DAKO Corporation, CA, USA). The specimens were examined with Olympus BX standard microscope and photographed on Kodak Ektachrome 64T film. The intensity of the staining for the *P. gingivalis* thiol proteinases studied was estimated semiquantitatively and scored (0–3) under constant, standard settings at 100× magnification.

Polymerase chain reaction for detection of P. gingivalis

In eight cases (four patients, four controls) the presence or absence of *P. gingivalis* in periodontal tissues was confirmed by polymerase chain reaction (PCR). Tissue samples of 5–10 mg were rinsed with buffer and homogenized mechanically on ice in 50 μl of buffer and boiled for 5 min. *Porphyromonas gingivalis* was detected by hot start PCR method according to Wahlfors *et al* (Wahlfors *et al*, 1995). Primers specific for *P. gingivalis* 16S rRNA (5′-GCG TAT GCA ACT TGC CTT AC-3′ and 5′-GTT TCA ACG GCA GGC TGA AC-3′) were used for the detection of the bacterium. Briefly, samples were centrifuged at 2100 *g* for 1 min, and 5 μl aliquots of the supernatants were added to the PCR reaction mixture, final volume 50 μl. *Porphyromonas gingivalis* ATCC 33277 was used as the positive control. DNA polymerase (Dynazyme II, Finnzymes, Espoo, Finland) was used for amplification with the reaction buffer (50 mM KCl, 10 mM tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP). The PCR amplification was performed with DNA thermal Cycler 480 (Perkin-Elmer Corporation, Boston, MA, USA). Before the PCR cycles the reaction components without the enzyme were kept at 96°C for 1 min and cooled to 80°C, at which temperature DNA polymerase (DynaZyme, FinnZymes, Espoo, Finland) was added to each tube. The PCR products were visualized by UV light after electrophoresis on agarose gel containing ethidium bromide.

Results

Biopsies of diseased gingiva were obtained from 13 patients with moderate to severe CP. Immunohistochemical analysis of the periodontal tissues revealed positive staining for *P. gingivalis* thiol proteinase in 11 of the 13 patients (85%) (Table 1). Positive staining was mainly located intracellularly in the periodontal epithelial cells (Figure 1a–d). Positive intracellular staining could be detected throughout the whole depth of both pocket and oral epithelium. The staining was more prominent in the oral epithelium in contrast to the junctional or pocket epithelia. The granular intracellular staining seemed to locate in the perinuclear region of the cytoplasm (Figure 1c,d). In the superficial epithelial cell layers some extracellular staining could also be detected (Figure 1b,c). In two cases (15%) positive staining was also detected in the underlying connective tissue. Strong immunoreactivity was detected by both primary antibodies used. Positive staining was not detected in the control specimens (infected gastric mucosa, cysts, granulomas and pancreas and conjugate controls; Figure 1e–g).

Subgingival plaque samples were collected from deep suppurating periodontal pockets of all CP patients. Samples were cultured and *P. gingivalis* was isolated from six individuals (46%). Gingival biopsies of all six patients found to be culture-positive for *P. gingivalis* were also found to show positive staining for *P. gingivalis* thiol proteinase.

Table 1 Immunohistochemical detection of *Porphyromonas gingivalis*-bacteria in tissue sections *in vivo*

Specimen	*Structure*	*Number of samples with positive staining*	*Average intensity of staining (0–3)*
AP ($n = 13$)	Oral epithelium	11 (85%)	1.82
	Pocket epithelium	11 (85%)	1.73
	Connective tissue	3 (23%)	0.27
Control gingiva ($n = 2$)	Oral epithelium	0	0
	Pocket epithelium	0	0
	Connective tissue	0	0
Other controls ($n = 13$)	Corpus ($n = 3$)	0	0
	Periapical granuloma ($n = 7$)	0	0
	Keratocyst ($n = 1$)	0	0
	Follicular cyst ($n = 1$)	0	0
	Pancreas ($n = 1$)	0	0

In eight cases (four patients, four controls) the presence or absence of *P. gingivalis* in periodontal tissues was analysed by PCR. In all six culture and staining -negative cases (two patients, four controls) the absence of *P. gingivalis*-infection was confirmed by PCR. The presence of *P. gingivalis*-infection of two CP patients who were culture-negative but stained positive for *P. gingivalis* was also confirmed with PCR (Figure 2).

Discussion

Porphyromonas gingivalis infection causes a strong inflammatory reaction in the periodontal tissues since it promotes the expression of a wide spectrum of inflammatory mediators and tissue destructive proteolytic cascades. Neutrophils accumulate in the periodontium together with macrophages, lymphocytes and plasma cells. Both local and circulating antibodies have been demonstrated in infected patients. All the machinery needed for immune defence thus seems to be present, but still a spontaneous recovery is rare and chronic *P. gingivalis* infection persists for years (Kinane *et al*, 1999). A number of virulence factors mediating host cell adherence and tissue destruction have been described. Host cell invasion has been suggested to be a possible immune evasion mechanism for *P. gingivalis*. Intracellular localization of *P. gingivalis* has been described *in vitro* by independent research groups (Lamont *et al*, 1992; Sandros *et al*, 1994; Deshpande *et al*, 1998). Recently, intracellular localization has also been demonstrated on buccal epithelial cells both *in vitro* and *in vivo* (Rudney *et al*, 2001). In the present study we demonstrate intracellular localization of *P. gingivalis* in gingival tissues of CP patients *in vivo*.

The thiol metalloproteinase immunohistochemically detected in the present study is a bacterial outer membrane protein specific for *P. gingivalis* and is not expressed by human cells (DeCarlo *et al*, 1997). The *P. gingivalis* proteinase was mainly detected intracellularly in the gingival epithelia and the localization of the granular staining was similar to that described in the *in vitro* studies. The *in vivo* granular intracellular staining seemed to locate in the perinuclear region of the cytoplasm and was surrounded by a thiol proteinase cloud corresponding to the *in vitro* findings by Belton *et al* (1999). It was not detected in the control samples and only in a proportion of the CP patient samples. Therefore, the intracellular localization of the thiol proteinase can, at least partially, be explained by intracellular localization of the microbe itself. The intracellular staining was more prominent in the oral epithelium in contrast to the junctional or pocket epithelia. The latter two are much thinner and have a much shorter cell cycle and may not, therefore, provide a suitable focus for the chronic infection. It appears that the periodontal infection caused by *P. gingivalis* is not limited to the gingival pocket and adjacent tissues.

In the more superficial layers of the epithelium the enzyme was occasionally detected extracellularly, as well. Extracellular staining may be due to the extracellular localization of the microbe or the antigen detected. In some previous *in vivo* studies where *P. gingivalis* has been reported to penetrate into periodontal tissues, the infection has been described to mainly localize within intercellular spaces (Saglie *et al*, 1988; Hillmann *et al*, 1998). The *P. gingivalis* thiol proteinase detected in the present study may contribute to the adherence of the bacterium to the epithelial cells, which may partly explain the extracellular staining in the superficial layers (Kuramitsu *et al*, 1997; Grenier 1992). More superficial cells are also more prone to the diffusion of the enzyme into the epithelium from the surface-growing bacteria. Alternatively, superficial and aged epithelial cells are susceptible to leakage of their intracellular proteins intercellularly, including the *P. gingivalis* thiol proteinase.

In the present study *P. gingivalis* infection could not be detected by bacterial culture in six cases (55%) of 11 found to be positive by immunohistochemistry or PCR. This may be because of the fact that the sensitivity of anaerobic bacterial culture is known to be moderate at its best. Most anaerobes tolerate transportation poorly and are sensitive to indoor oxygen during plating and microbiological analysis. For the immunohistochemical or PCR analyses the microbes do not need to be viable and the method is thus less susceptible to

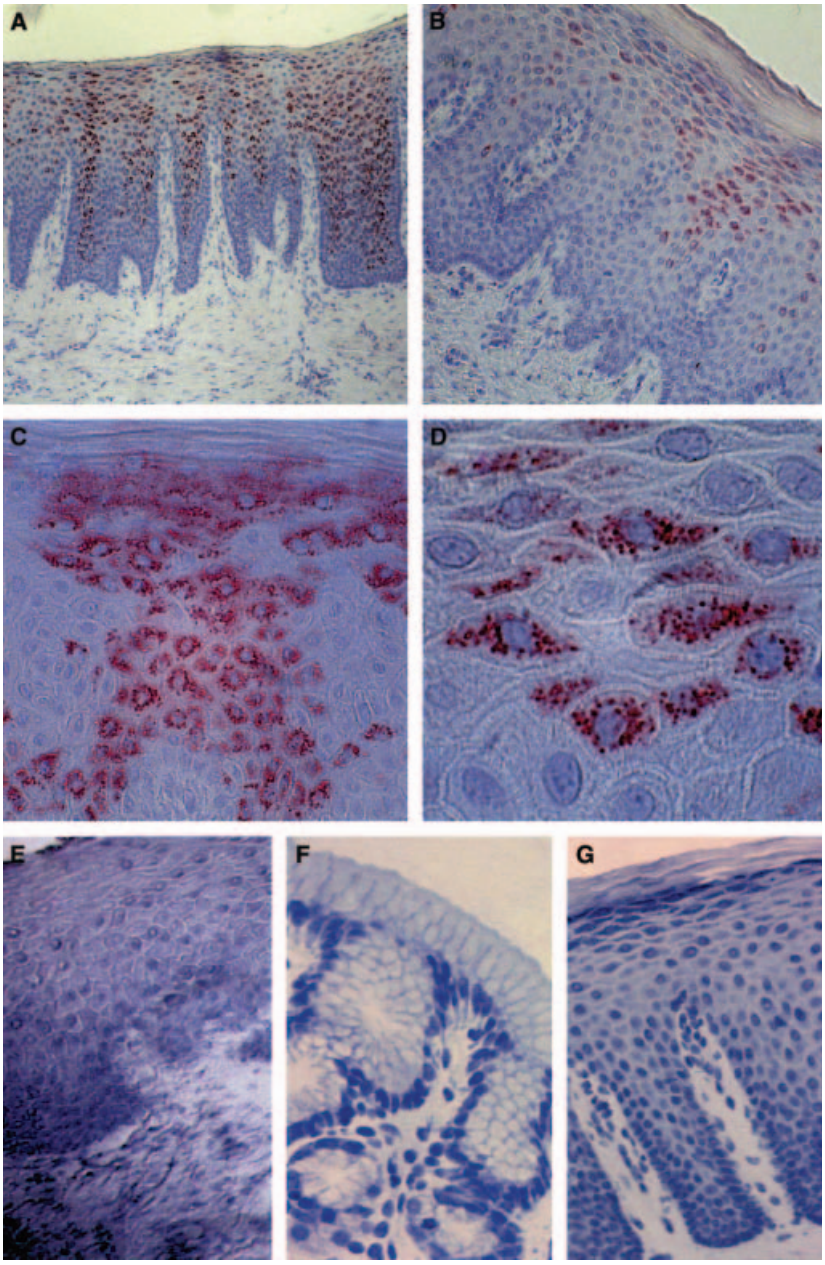

Figure 1 Intracellular localization of *Porphyromonas gingivalis* thiol proteinase. (**a**) *Porphyromonas gingivalis* thiol proteinase is expressed throughout the oral epithelium as detected by the mouse monoclonal antibody IIB_2. (**b**,**c**) The *P. gingivalis* thiol proteinase is mainly located in the epithelium whereas the underlying connective tissue is mostly negative for the thiol proteinase as detected by the mouse monoclonal antibody IIB_2. The *P. gingivalis* thiol proteinase is expressed intracellularly and can be seen extracellularly only in the superficial layer of aged cells. The overlaying keratin layer mainly stains negative. (**d**) *Porphyromonas gingivalis* thiol proteinase is expressed inside epithelial cells as detected by the mouse monoclonal antibody VA_1. The staining is granular indicating of intracellular localization of *P. gingivalis* bacteria. Controls: control stainings were performed on *P. gingivalis* culture negative healthy oral epithelium (**e**), *Helicobacter pylori*-infected gastric corpus (**f**) and on *P. gingivalis*-infected oral epithelium by omitting the primary antibody (**g**). (Original magnifications, **a**, 100×; **b**, 160×; **c**, 400×; **d**, 1000×, **e-g**, 160×)

postsampling interferences. In the present study the sensitivity of immunohistochemistry was found to be similar to that of PCR. It is also possible that the intracellular localization of the *P. gingivalis* represents a latent phase of the infection where the number of culturable bacteria in the gingival pocket is very low. Bacterial latency is well documented for other intracellular bacteria *e.g. Bacillus tuberculosis* (Casadevall and Pirofski, 2000; Pfyffer *et al*, 2003).

Porphyromonas gingivalis can alone or together with other potential periodontopathogens induce degranulation and activation of MMPs from neutrophils even without opsonization (Ding *et al*, 1997). In addition, the thiol proteinases produced by *P. gingivalis* are known to up-regulate the production and release of cytokines and other pro-inflammatory activators of cellular second-messenger stimuli. It has also been shown that certain *P. gingivalis* cysteine proteinases capable of hydrolysis and inactivation of IL-12 could result in local interferon (IFN)-γ accumulation and thus affect the Th1 and Th2 T-cell phenotype in periodontitis (Yun *et al*, 2001). We have previously shown that the enzyme detected in the present study promotes the release and activation of MMPs by cultured human gingival fibroblasts (Uitto *et al*, 1989; DeCarlo *et al*, 1998; Chen *et al*, 2000). However, the consequences of the expression of this powerful enzyme inside epithelial cells needs further study. The *P. gingivalis* thiol proteinase may amend or interfere with the activity of antimicrobial peptides produced by the epithelium and thus contribute to the

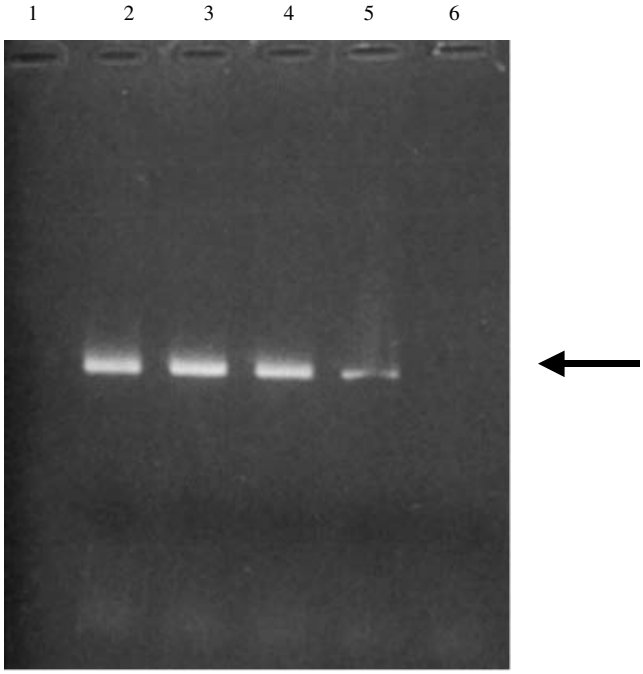

Figure 2 Ethidium bromide-stained agarose gel showing positive and negative results of PCR amplification of the *Porphyromonas gingivalis* 16S rRNA (arrow). An image of a representative gel of three patient samples, and positive and negative controls. Lanes: 1, sample from a CP patient negative for *P. gingivalis* in immunohistochemistry; 2–3, sample from a CP patient positive for *P. gingivalis* in immunohistochemistry; 4, sample from another CP patient positive for *P. gingivalis* in immunohistochemistry; 5, *P. gingivalis* ATCC 33277 positive control; 6, *Helicobacter pylori*-infected gastric biopsy

P. gingivalis evasion of the host defence systems (Darveau *et al*, 1998; Dale, 2002).

Streptococcus pneumoniae, like *P. gingivalis* is also usually considered to be an extracellular pathogen. A clinical study by Talbot et al documented the persistence of pneumococcus in the middle-ear fluid of patients treated with several different antibiotics (Talbot *et al*, 1996). *In vitro* studies have indicated that pneumococci can enter and survive inside *e.g.* human lung alveolar carcinoma cells, a mechanism which has been suggested to contribute to the persistence of the otherwise susceptible microbe (Brook and Gober, 1998). Diseases caused by mainly extracellular pathogens respond to therapy with antimicrobial agents such as beta-lactam antibiotics clearly. Many of these antibiotics penetrate through the cell membranes poorly. However, if the pathogen has the capability to reside intracellularly it may be advantageous to destroy organisms in intracellular sites, as well, to reduce relapse, recolonization, and possibly the development of resistance. In a recent *in vitro* study the activities of antimicrobial agents against intracellular pneumococci was studied (Mandell and Coleman, 2000). Gentamicin, penicillin G and rifampicin resulted in significant survival of the pneumococci inside the cells whereas all macrolides and fluoroquinolones studied were found effective.

Ability to invade host cells is an efficient mechanism for any microbe to evade host immune systems. Intracellular localization protects the microbe also from many antimicrobial treatments. The demonstration of the ability of *P. gingivalis* to enter host gingival epithelial cells *in vivo* raises new questions of the exact localization of the chronic infection, transmission of the microbe as well as of pathogenesis and treatment guidelines of periodontitis. Intracellular localization of *P. gingivalis* partly explains why the eradication of the microbe by conservative mechanical means is often difficult if not impossible. In fact, mechanical cleaning may even result in increased spreading of the microbe to deeper tissues and blood circulation. The choice of antibiotics also needs to be re-evaluated as treatment with drugs like beta-lactams that do not enter the host cells may even favour the survival of *P. gingivalis*.

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