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ORIGINAL ARTICLE

Stimulatory response of neutrophils from periodontitis patients with periodontal pathogens

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OBJECTIVE: Neutrophils play a crucial role in the defense of invading bacteria by releasing biologically active molecules. The response of peripheral blood neutrophils was studied in periodontitis-affected patients and in healthy controls towards stimulation to *Porphyromonas gingivalis* (*Pg*) and *Actinobacillus actinomycetemcomitans* (*Aa*) extracts.

MATERIALS AND METHODS: Peripheral venous blood was drawn from 23 adult patients with moderate to advanced chronic periodontitis (probing depth ≥5 mm, attachment loss ≥3 mm), and 30 healthy volunteers. Neutrophil response followed by metalloproteinase-9 (MMP-9) and interleukin-8 (IL-8) secretion was assayed by zymography and enzyme-linked immunosorbent assay, respectively, on both whole blood and purified neutrophils. In addition to periodontal pathogen extracts, known stimulating agents were tested, such as *Escherichia coli*-lipopolysaccharide (LPS), phytohemagglutinin, and zymosan A.

RESULTS: Neutrophil response, expressed as a secretion ratio under stimulated and non-stimulated conditions, measured in whole blood, showed no differences between periodontitis and healthy controls. Instead, in purified neutrophils from patients, MMP-9 exhibited a significantly higher secretion ratio with LPS and *Pg* (1.5- to 2-fold), whereas IL-8 showed a larger increase in secretion ratio (3- to 7-fold) in the presence of *Pg*, *Aa*, LPS, and zymosan A.

CONCLUSION: Peripheral neutrophils of periodontitis-affected patients are more reactive as suggested by their significantly higher response toward periodontal pathogen extracts and other stimulating agents.
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Introduction

Periodontitis is a chronic inflammatory disease characterized by connective tissue and alveolar bone destruction, eventually leading to tooth loss. The two major pathogens in periodontal disease are the Gram-negative bacteria *Porphyromonas gingivalis* (*Pg*) and *Actinobacillus actinomycetemcomitans* (*Aa*) (Socransky *et al*, 1998; Eley and Cox, 2003). These microorganisms induce the release of cytokines from fibroblasts and inflammatory cells (Yoshimura *et al*, 1997; Johansson *et al*, 2005). Neutrophils, macrophages, B and T lymphocyte cells accumulate in periodontal lesions and tissue destruction takes place (Assuma *et al*, 1998; Gamonal *et al*, 2001). The microorganisms seem to produce destruction directly through their endotoxins (LPS) and indirectly through activation of the host cells to produce a variety of biologically active substances, i.e. cytokines, arachidonic acid metabolites, and proteolytic enzymes (Trevani *et al*, 2003). *Pg* stimulates the expression of interleukin-8 (IL-8) in peripheral neutrophils (Yoshimura *et al*, 1997; Sugita *et al*, 1998). IL-8 is a potent cytokine that leads to the induction of chemotaxis, migration, and activation of neutrophils (Kuhns *et al*, 1998; Godaly *et al*, 2001).

When exposed to bacteria-derived products or inflammatory mediators, neutrophils respond by chemotaxis, phagocytosis, and microbial killing through oxygen radical- and non-oxygen-dependent mechanisms (Kobayashi *et al*, 2003; Moraes and Downey, 2003). During phagocytosis, neutrophils produce reactive oxygen species (ROS), together with the release of cytotoxic granule components (Kobayashi *et al*, 2003). Gelatinase and specific granules are reservoirs of metalloproteinases (MMPs), while azurophil granules contain a neutral protease called elastase (Faurschou and Borregaard, 2003; Uitto et al, 2003).

In response to stimulation, the gelatinase granules are more easily exocytosed followed by specific granules. The former contain MMP-9, or gelatinase B as well as leukolysin or MMP-25; these MMPs occur in inactive form, undergoing proteolytic activation following exocytosis. The latter, namely, specific granules, contain MMP-8 and some smaller amounts of gelatinase as well. On the other hand, azurophil granules undergo limited exocytosis in response to stimulation (Faurschou and Borregaard, 2003; Uitto et al, 2003).

Microbial infection is detected by the family of innate immune signaling receptors known as the Toll-like receptors (TLRs) present in neutrophils, that recognize conserved microbial structures called pathogen-associated molecular patterns (Akira, 2003; Kopp and Medzhitov, 2003). CR3 and Fc gamma Rs are receptors involved in the phagocytic process leading to bacterial destruction by the production of reactive oxygen intermediates and degranulation (Forsberg et al, 2001; Kobayashi et al, 2003).

Activation of neutrophils and the cleavage of cell matrix molecules by MMP-8, MMP-9 and elastase, generate peptide fragments that are chemotactic for monocytes, promoting the recruitment of inflammatory cells to the site of injury and thus releasing proinflammatory mediators that amplify the local inflammatory response (Faurschou and Borregaard, 2003; Figueredo et al, 2005). The role of neutrophils in the pathogenesis of periodontal disease is unclear. The protective functions of neutrophils have been studied in relation to defense mechanisms against periodontal pathogens (Kobayashi et al, 2003). However, this increased neutrophil activity could be responsible for the tissue destruction observed in periodontitis (Kantarci and Van Dyke, 2002; Fredriksson et al, 2003; Kantarci et al, 2003; Zekonis and Zekonis, 2004). Deguchi et al (1990) showed that LPS-stimulated human neutrophils get adhered to damaged periodontal tissue.

The sites of chronic periodontitis are rich sources of virulence agents, such as LPS, proteases from *Pg* and a leukotoxin produced by *Aa* that induces neutrophil degranulation (Geerts et al, 2002; Deas et al, 2003; Johansson et al, 2005). Reports on healthy subject neutrophils described an increased activity of MMP-9 and MMP-8, followed by stimulation with *Pg* or *Aa* (Ding et al, 1997; Claesson et al, 2002). In another report, the stimulation of neutrophils with LPS from *Escherichia coli* and *Aa* showed the release of significantly greater amounts of IL-8 (Yoshimura et al, 1997).

The effects of periodontal pathogen extracts and some known stimulating agents were studied on peripheral whole blood and on purified neutrophils of both control and periodontitis-affected patients. The effects were followed by (a) MMP-9 release from gelatinase granules required during neutrophil extravasations (Faurschou and Borregaard, 2003) and (b) IL-8 release, one of the most abundant cytokines produced by neutrophils, reported in high amounts in the plasma of rapidly progressive periodontitis patients, which suggests a systemic effect of this periodontal disease (Gainet et al, 1998).

Materials and methods

The study group comprised 23 adult patients with moderate to advanced chronic periodontitis selected from the Center of Diagnostic and Treatment of Northern Metropolitan Health Services. The criteria for entry were a minimum of 14 natural teeth, excluding third molars, and including at least 10 posterior teeth. Patients with chronic periodontitis had moderate to advanced periodontitis (at least five to six teeth had sites with probing depth (PD) ≥5 mm and with attachment loss ≥3 mm and extensive bone loss in radiography), and had received no periodontal treatment at the time of examination. Subjects did not suffer from systemic illness and had received no antibiotics or non-steroid anti-inflammatory therapy in the 6-month period prior to the study. The control group comprised 30 subjects with no clinical signs of periodontal [absence of clinical attachment loss (CAL) or increased probing pocket depths] and systemic diseases. Prior to the study, all the subjects received supragingival prophylaxis to remove gross calculus and allow PD. The protocol was clearly explained to all patients and controls, and Institutional Review Board-approved informed consents were obtained. The protocol stated that, within 2 weeks of the detection of disease activity, all the patients would be provided with periodontal treatment. Periodontal therapy consisted of scaling, root planing, and instruction in oral hygiene.

Clinical measurements

Clinical parameters were evaluated by a single calibrated investigator in all teeth (excluding third molars) and included PD, CAL and dichotomous measurements of supragingival plaque accumulation or plaque index (PI) and bleeding on probing to the base of the crevice (BOP). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual positions. The Florida Disk Probe was used for relative attachment level recordings and the Florida Probing Depth for PD recording (Florida Probe Corporation, Gainesville, FL, USA).

Whole blood/purified neutrophil stimulation assay

Whole blood from healthy human volunteers and periodontitis-affected patients was collected on the day of the assay, and was drawn into heparinized syringes (10 U ml^{-1} heparin sulfate; Liquemine; Roche Pharma, Reinach, Switzerland). Neutrophils were purified from whole blood samples as described previously (Rose et al, 1992), using a Hystopaque 1077 gradient (Sigma Chemical Co, St Louis, MO, USA), and were diluted to 10^6 cells ml^{-1} in RPMI 1640 with 10% bovine fetal serum (Gibco, Basel, Switzerland), 2 mM glutamine and 5 μg ml^{-1} streptomycin. Before assay, 1 ml of whole blood was diluted 1:4 with RPMI 1640 containing 10% bovine fetal serum or 1 ml purified neutrophils was incubated at 37°C for 1 h. Assays were started by the addition of up to 10% of the assay volume of the stimulating agents previously diluted in phosphate-buffered saline (PBS; 0.137 M NaCl, 12 mM Na_2HPO_4,2 mM KH_2PO_4 adjusted to pH 7.4); assays were then maintained at 37°C for 1 h. Different concentrations of stimulatory agents were initially tested in order to choose appropriate conditions, which include both neutrophil viability and stimulatory effect. Chosen concentrations were in the range of those used by other authors (Pugin *et al*, 1999; Hayashi *et al*, 2003). *Escherichia coli* LPS (O111:B4) (tests ranging from 0.05 to 0.8 $\mu g\ ml^{-1}$) was used at a final concentration of 0.8 $\mu g\ ml^{-1}$, phytohemagglutinin (PHA; 1.0–5.0 $\mu g\ ml^{-1}$) at 2.0 $\mu g\ ml^{-1}$ and zymosan A (5.0–100 $\mu g\ ml^{-1}$) at 100 $\mu g\ ml^{-1}$ (all were from Sigma Chemical Co). *Pg* and *Aa* extracts (prepared in our laboratory) were used at 0.36 and 0.07 $\mu g\ ml^{-1}$, respectively. Tested dilutions of bacterial extracts were in the range of 0.09–0.360 $\mu g\ ml^{-1}$ for *Pg* and 0.02–0.07 $\mu g\ ml^{-1}$ for *Aa*. Concentrations used were the maximum allowed for the viability of the neutrophils. Addition of PBS alone was used as a non-stimulated condition. After incubation cell suspensions were spun at 2000 *g* for 10 min, and the supernatant of blood/neutrophil samples was stored at −20°C until use.

Preparation of bacterial extracts
Actinobacillus actinomycetemcomitans (Y4) was grown in enriched tryptic soy agar and tryptone soy broth containing Na_2CO_3, L-cysteine, hemin and vitamin K. *Porphyromonas gingivalis* (A7A1-28) was cultured in enriched Todd Hewitt broth and butylated hydroxyanisole containing the same supplements. Anaerobic bacteria were cultured under strictly anaerobic conditions according to Loomer *et al* (1995). After culturing, the bacteria were killed by 10 min boiling, then washed thrice with PBS, and stored at −20°C. Boiling does not destroy the LPS component. Amounts of bacterial extracts were expressed according to their protein content measured by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions.

Gelatin zymography
Gelatin zymography was performed according to Pozo *et al* (2005). Whole blood or purified peripheral neutrophils were resuspended in non-reducing sodium dodecyl sulfate (SDS) sample buffer (0.4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 0.05% bromophenol blue); boiling was not permitted to allow further enzyme reactivation, and loaded onto a 10% SDS–polyacrylamide gel, including 1 $mg\ ml^{-1}$ of gelatin (Merck, Darmstadt, Germany). In order to work in a linear zone of gelatinase activity, adequate conditions for sample dilution and volume were tested. The best conditions were: sample dilution 1:20 in upper SDS-PAGE buffer (Tris-HCl, pH 6.8), and a volume of 5 μl of the diluted samples previously treated with the non-reducing Laemmli buffer (Pozo *et al*, 2005). After the electrophoretic run, SDS was removed from the gel before incubation for 20 h at 37°C with the renaturating buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3, pH 7.5). After Coommassie brilliant blue R-250 staining, and destaining (7% acetic acid) (Ejeil *et al*, 2003), proteolysis areas were estimated by densitometric analysis using the 'Uni-Scan-it Gel-Digitizing System' software (Silk Scientific Corporation, Orem, UT, USA). The arbitrary density units (in pixels) of the stimulating conditions were referred to as the basal or non-stimulated condition. Through zymography, both MMP and proMMP (non-enzymatically active form) can be detected because the presence of SDS in the gel and its later removal through Triton X-100 exchange activates zymogen without changing the molecular weight (Pugin *et al*, 1999).

Determination of IL-8 secretion upon stimulation
Interleukin-8 was measured in 50–100 μl portions of an incubation medium with an enzyme-linked immunosorbent assay kit (BioSource Europe S. A., Nivelles, Belgium) following the manufacturer's instructions.

Statistical analysis
When necessary (IL-8 results), data from patient and control groups were normalized using the logarithmic function. The differences between values of both groups were assessed by a two-tailed Student's *t*-test, using the statistic software Systat 11.0. Statistical significance was set at a value of $P < 0.05$.

Results

Clinical characteristics of all the 23 patients under study are shown in Table 1. We used whole blood from 13 patients and purified neutrophils from the remaining 10. The mean values ± s.d. of PI, PD, BOP, and CAL are significantly higher than those of healthy individuals who exhibited PD < 3 mm, no attachment loss, and no clinical inflammation.

A representative gelatin zymogram of purified neutrophil secretion from a periodontitis-affected patient stimulated with LPS, PHA, *Pg*, *Aa*, and zymosan A, together with the non-stimulated condition (lane 1), and the presence of a positive standard of proMMP-9 is shown in Figure 1a, where gelatinolytic zones are visualized as white bands on a dark background. In Figure 1b we compare the effect of the stimulating agents on both a patient (lanes 1–5) and a healthy

Table 1 Clinical characteristics of periodontitis and healthy groups (mean ± s.d.)

	Periodontitis group (n = 23)	*Healthy group (n = 30)*
Age (years)	36.69 ± 9.49	38.42 ± 8.42
Women (*n*)	17	21
Mean probing depth (mm)	3.35 ± 0.55*	1.65 ± 0.48*
Mean attachment level (mm)	3.30 ± 0.57*	0.26 ± 0.26*
% sites with plaque	61.34**	25.60**
% sites with bleeding on probing	51.87*	1.42*

*$P < 0.0001$; **$P < 0.001$.

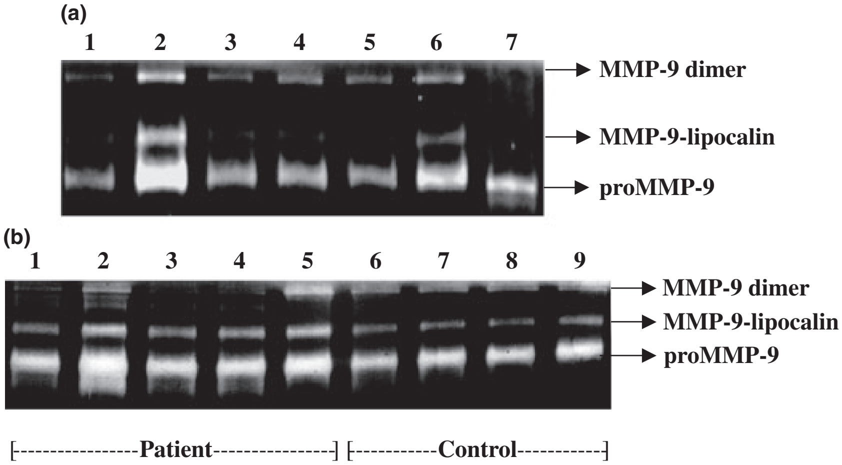

Figure 1 Zymogram of various forms of metalloproteinase-9 (MMP-9) released from active neutrophils. (**a**) Neutrophil sample from a periodontitis-affected patient. Lane 1: neutrophils without stimulation; lane 2: neutrophils stimulated with lipopolysaccharide (LPS; 0.8 μg ml^{-1}); lane 3: neutrophils stimulated with phytohemagglutinin (2 μg ml^{-1}); lanes 4 and 5: neutrophils in the presence of *Porphyromonas gingivalis* (*Pg*; 0.36 μg ml^{-1}) or *Actinobacillus actinomycetemcomitans* (*Aa*; 0.07 μg ml^{-1}), respectively; lane 6: neutrophils stimulated with zymosan A (100 μg ml^{-1}); and lane 7: a positive proMMP-9 control. (**b**) Neutrophil sample from a patient (lanes 1–5). Lane 1: without stimulation; lane 2: stimulated with LPS (0.8 μg ml^{-1}); lane 3: stimulated with *Pg* (0.36 μg ml^{-1}); lane 4: stimulated with *Aa* (0.07 μg ml^{-1}); lane 5: stimulated with zymosan A (100 μg ml^{-1}). Neutrophil sample from a healthy subject (lanes 6–9) – lane 6: without stimulation; lane 7: stimulated with LPS (0.8 μg ml^{-1}); lane 8: stimulated with *Pg* (0.36 μg ml^{-1}); lane 9: stimulated with zymosan A (100 μg ml^{-1}). MMP-9 dimer (175 kDa), MMP-9-lipocalin (130 kDa), and proMMP-9 (92 kDa)

control sample (lanes 6–9). The 92 kDa band corresponds to proMMP-9, a 130-kDa band to the reported neutrophil–MMP-9–lipocalin complex (Westerlund *et al*, 1996), and a gelatinolytic band over 175 kDa, described as the dimeric form of an MMP-9 (Olson *et al*, 2000). No active form for MMP-9 was detected because of the absence of gelatinolytic activity at 84 kDa. Whole blood samples after incubation showed a similar pattern.

Measurements of the proMMP-9 band gave more reproducible results than when also including the other two MMP-9-related bands (lipocalin complex and dimer). The stimulating agents did not produce a significant effect on whole blood in both healthy and patient groups. Results with purified neutrophils from patients with either moderate or advanced periodontitis were not statistically different, so all the results were aggregated. To compare the effect of these agents on the secretory response, data were expressed as the secretion ratio of the gelatinolytic area under stimulated and non-stimulated conditions (S-proMMP-9/N-proMMP-9). Figure 2 shows that purified neutrophils from patients display significantly higher levels of proMMP-9 than those of controls ($P < 0.05$), between 1.5- and 2-fold, using LPS or Pg treatments. The dispersion of the results with zymosan A hinders significant statistical differences (Figure 2).

Similar to MMP-9 results, IL-8 release from the healthy group samples (whole blood and purified neutrophil samples) was not increased by the stimulating agents. IL-8 secretion promoted by these agents in whole blood samples did not show differences between both groups (data not shown). Instead, in the purified neutrophils of periodontitis-affected patients, LPS, *Pg*, *Aa*, and zymosan A triggered a significant response to

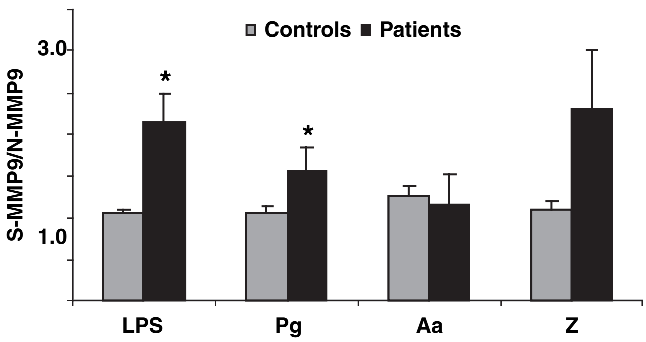

Figure 2 Comparison of the effect of stimulating agents on metalloproteinase-9 (MMP-9) secretion from purified neutrophils of periodontitis-patients and controls. Results are expressed as the secretion ratio under stimulated and non-stimulated conditions (S-MMP-9/N-MMP-9). Stimulant concentrations are similar to those mentioned in Figure 1. Data correspond to mean values ± standard error of the mean (s.e.m.). *$P < 0.05$ between patients and healthy controls

IL-8 release when compared with the non-stimulated condition (S-IL-8/N-IL-8) (Figure 3a).

Unlike the healthy group, the level of secreted IL-8 (expressed as pg ml^{-1}) for the patient group was significantly lower either in the presence or absence of stimulating agents (Figure 3b). These results differ from those obtained for proMMP-9 secretion from gelatinase granules, where no differences were observed in proMMP-9 levels for both groups in the non-stimulated condition (incubation with PBS).

Discussion

The enhancement of proMMP-9 (1.5- to 2-fold) and of IL-8 (3- to 7-fold) secretion under the stimulated

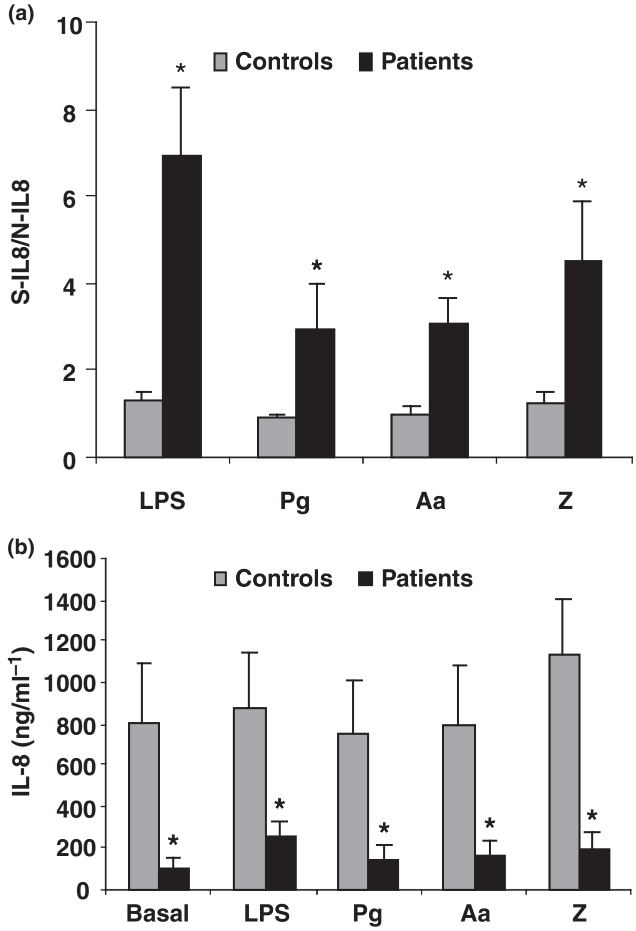

Figure 3 Comparison of the effect of stimulating agents on interleukin-8 (IL-8) secretion from purified neutrophils of periodontitis-patients and controls. (**a**) Ratio of IL-8 secretion in stimulated/non-stimulated conditions (S-IL-8/N-IL-8). (**b**) Levels of IL-8 secreted from neutrophils. Data are expressed as mean values ± s.e.m. Stimulant concentrations are similar to those mentioned in Figure 1. $*P < 0.05$ between patients and healthy controls

condition by periodontal pathogen extracts and other proinflammatory mediators suggests that neutrophils are more reactive in periodontitis-affected patients than in controls. Purified neutrophil samples proved to be more adequate than whole blood samples for neutrophil stimulation as they helped in detecting differences between the patients and controls. This hyper-reactivity may be secondary to the inflammatory reaction occurring in the gingival tissue during periodontal disease; however, such hyper-reactivity could also be the result of constitutive differences in neutrophils from controls and periodontitis patients. This latter situation could be of relevance in the pathogeny of the periodontal disease (Fredriksson *et al*, 2003; Gustafsson *et al*, 2006); hence it seems important to determine whether the hyper-reactivity followed by proMMP-9 and IL-8 release is reverted after non-surgical periodontal treatment. Gainet *et al* (1998) reported normalization of IL-8 concentration in plasma from progressive periodontitis patients after treatment, and we observed a reduction of MMP-activity to control values in gingival crevicular fluid after a 3-month treatment (Pozo *et al*, 2005).

The significantly reduced levels of IL-8 secretion in periodontitis-affected patients could be attributed to the known endotoxin tolerance phenomenon (West and Heagy, 2002). Marie *et al* (1998) observed a significantly decreased release of IL-8 in neutrophils from patients with infectious and non-infectious systemic inflammatory response syndrome; the observed decrease in periodontitis patients did not, however, reflect tolerance because neutrophils were not desensitized to a second challenge. The downregulation of the synthesis and release of excessive proinflammatory cytokines after infection could be an adaptive mechanism to avoid deleterious effects for the host. Plasma samples from periodontitis-affected patients showed significantly reduced systemic levels of IL-1β compared with healthy controls (Johansson *et al*, 2005).

Different forms of MMP-9 (proMMP-9, MMP-9-lipocalin complex, and a dimeric form) are released after 60 min incubation either from blood or purified neutrophils. This gelatinase activity can be attributed to neutrophils and monocytes/macrophages. A secretion within 60 min cannot be the result of *de novo* protein synthesis by monocytes/macrophages, requiring at least 7 h of incubation, hence this secretion should come from neutrophil granules (Pugin *et al*, 1999). In addition, the presence of the 130 kD proMMP-9–lipocalin complex in non-reducing SDS-PAGE zymography indicates a PMN origin for this gelatinase (Olson *et al*, 2000; Faurschou and Borregaard, 2003). A report on interaction between neutrophils and whole cells of *P. gingivalis* and other periodontopathogens showed that azurophil, specific, and gelatinase granules are triggered during this interaction (Ding *et al*, 1997).

Elevation of systemic inflammatory mediators including C-reactive protein, IL-6, neutrophils, and bacterial endotoxins occurs in patients with periodontitis (Loos *et al*, 2000; Geerts *et al*, 2002; Deas *et al*, 2003; Johansson *et al*, 2005). This suggests that the infected periodontum serves as a source of circulating endotoxin. Low levels of endotoxin or bacterial products derived from periodontitis may therefore, contribute to immune activation and could be associated with general systemic conditions such as miscarriage or cardiovascular disease (Farell *et al*, 2006; Spahr *et al*, 2006; Taylor *et al*, 2006). In this disease the persistent accumulation of bacteria in the periodontal pocket maintains the recruitment of inflammatory cells and a prolonged and sustained release of inflammatory mediators (Eley and Cox, 2003; Sela *et al*, 2003; Pozo *et al*, 2005). These products can amplify the local inflammatory response, further promoting leukocyte and platelet recruitment and thus perpetuating the inflammation. Additionally, they can lead to elevated amounts of these mediators into the bloodstream.

As the factors released by activated neutrophils are potentially toxic to host tissues, neutrophils are subsequently removed by the process of apoptosis (Moraes and Downey, 2003; Scheel-Toellner *et al*, 2004). This mechanism prevents the secretion of pro-inflammatory mediators into the tissues avoiding the inflammatory response. Thus, modulation of the activation status of the neutrophils is essential in determining the balance between the immune defense and host injury (Kobayashi *et al*, 2003; Moraes and Downey, 2003). MMPs and other enzymes released from activated neutrophils modify constituents of the extracellular matrix (ECM) through which cells migrate to reach target organs (Vaday and Lider, 2000). Growing evidence suggests that mediators such as MMPs and cytokines secreted from immune cells invading into tissues through ECM induce distinctive cellular responses, being probably part of a regulatory mechanism of perpetuation or arrest of inflammation (Vaday and Lider, 2000).

Neutrophils recognize bacterial structure mainly via the TLRs (Akira, 2003). TLRs involved in the recognition of pathogen-associated molecular patterns, activate intracellular signaling and transcription of protein essential for the induction of the inflammatory response and elimination of invading pathogens (Sabroe *et al*, 2003; Netea *et al*, 2004; Parker *et al*, 2005). It is quite probable that similar to *E. coli* LPS, the stimulating effects of *Pg*, *Aa*, and zymosan depend on their interaction with TLR-2 and/or TLR-4 (Armant and Fenton, 2002; Akira, 2003; Kopp and Medzhitov, 2003; Hashimoto *et al*, 2004). The response to zymosan A, a *Saccharomyces cerevisiae* cell wall particle, may occur through both the TLR-2 receptor (Hayashi *et al*, 2003), and complement C3bi (CR3) receptor for the opsonized particle of zymosan. TLRs detect structurally different components in pathogens. In *Pg* both receptors are involved: lipid A, a component of LPS is the main inmunomodulator acting through TLR-4, while the lipoprotein activates cells through a TLR-2-dependent pathway (Akira, 2003; Hashimoto *et al*, 2004). In chronic periodontitis, the gingival tissue is infiltrated by large numbers of TLR-2 and TLR-4 cells (Muthukuru *et al*, 2005). However, real-time PCR analysis showed a significant downregulation of TLR-2 and TLR-4 mRNA suggesting that the sustained exposure to bacterial structure modulates local immune response (Muthukuru *et al*, 2005). However, we have to bear in mind that in the boiling process used during the bacterial extracts preparation in this study, some important pathogenic agents, such as leukotoxin produced by *A. actinomycetemcomitans* are lost. This toxin is recognized by the lymphocyte function-associated antigen 1 receptor of leukocytes and induces the lysis of neutrophils and monocytes (Baehni *et al*, 1979; Yamaguchi *et al*, 2004).

Attempts to find the differential expression of either selected adhesion molecules or secretion of IL-4 and IL-6 in peripheral blood leukocytes in patients with various forms of periodontal diseases have been unsuccessful (Pietruska *et al*, 2004, 2005). Previous reports have studied only the stimulation of neutrophils with non-opsonized *E. coli*. However, *E. coli* LPS and opsonized *Staphylococcus aureus* demonstrated a significant increase in reactive oxygen metabolites release in periodontitis-affected patients with respect to healthy controls (Fredriksson *et al*, 2003; Zekonis and Zekonis, 2004; Sadzevieciene *et al*, 2005; Gustafsson *et al*, 2006). In addition, elastase release after stimulation with *S. aureus* has been reported to be significantly higher in patients in relation to the controls (Figueredo *et al*, 1999). Hence, the relevance of the current study lies in the demonstration of the hyper-reactivity of peripheral neutrophils in patients with advanced or moderate periodontitis toward periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* based on MMP-9 and IL-8 release.

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