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Neutrophils in chronic and aggressive periodontitis in interaction with *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*

Arndt Guentsch¹, Magdalena Puklo², Philip M. Preshaw³, Eike Glockmann¹, Wolfgang Pfister⁴, Jan Potempa², and Sigrun Eick⁴

¹Department of Conservative Dentistry, University Hospital, Friedrich-Schiller-University Jena, Germany ²Faculty of Biochemistry, Biophysics and Biotechnology, Department of Microbiology, Jagiellonian University, Krakow, Poland ³Department of Periodontology, School of Dental Sciences, University of Newcastle upon Tyne, UK ⁴Institute of Medical Microbiology, University Hospital, Friedrich-Schiller-University Jena, Germany

Abstract

AIM—The aim of this *in vitro* study was to study phagocytosis and killing of *Porphyromonas gingivalis* ATCC 33277 and *Aggregatibacter actinomycetemcomitans* Y4 by peripheral blood PMNs taken from aggressive and chronic periodontitis patients. Also, the release of reactive oxygen species (ROS) and human neutrophil elastase (HNE) upon the interaction of PMNs with bacteria was measured.

METHODS—Peripheral blood PMNs obtained from 12 chronic periodontitis patients, 6 aggressive periodontitis patients and 12 healthy controls were exposed to *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* following opsonisation of the bacteria using the patient's own serum. Phagocytosis and killing of the bacteria as well as the extracellular HNE activity were quantified for up to 2 hours. The total amount and the extracellular release of ROS were measured by luminol and isoluminol dependent chemiluminescence.

RESULTS—PMNs from chronic (62.16 ± 19.39 %) and aggressive periodontitis (43.26 ± 26.63 %) patients phagocytosed more *P. gingivalis* than the healthy controls (24.43 ± 19.87 %) at 30 mins after exposure to the bacteria ($p < 0.05$). PMNs from subjects with chronic and aggressive periodontitis released significantly more ROS and demonstrated greater HNE activity in the absence of any stimulus than PMNs from healthy controls ($p < 0.05$). The total release of ROS increased in chronic periodontitis PMNs and in the control group PMNs after interaction with *P. gingivalis*. The interaction with *A. actinomycetemcomitans* resulted in greater total ROS release in chronic periodontitis PMNs and in the control group PMNs than *P. gingivalis*.

CONCLUSION—PMNs in aggressive and chronic periodontitis are hyperactive even without any particular stimulus. The extracellular release of ROS and neutrophil elastase by PMNs may not

only affect bacterial virulence and/or viability, but also contribute to damage of the surrounding periodontal tissues.

Keywords

PMN; phagocytosis; ROS; aggressive periodontitis; chronic periodontitis; *Aggregatibacter actinomycetemcomitans*; *Porphyromonas gingivalis*

Introduction

Periodontitis is a chronic inflammation of the periodontal tissues in response to the presence of subgingival bacteria. *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are regarded as periodontal pathogens and are clearly associated with periodontal disease (1). These bacteria are found in patients with aggressive as well as chronic periodontitis (2), but patients with aggressive periodontitis are considered to have a higher load of *A. actinomycetemcomitans* (3), while *P. gingivalis* is more strongly associated with severe chronic periodontitis (4).

The host response to subgingival bacteria plays a critical role in periodontal pathogenesis (5). Polymorphonuclear neutrophils (PMNs) represent the first line of cellular host responses against bacteria in the gingival sulcus (5). The antimicrobial activities of PMNs include oxygen-dependent and oxygen-independent mechanisms (6). The oxygen-dependent pathway involves the production of reactive oxygen species (ROS), molecules which are capable of initiating periodontal tissue destruction (7). The production of ROS by PMNs is primarily focused towards bacterial killing, but extracellular release of ROS results in collateral damage of the surrounding tissues (8). Non-oxidative microbial killing relies on the contents of three cytoplasmic granule subsets, the azurophilic (primary), specific (secondary), and gelatinase granules (9). After fusing with phagosomes, these granules deliver antimicrobial proteins and peptides such as defensins, bactericidal/permeability-increasing protein, azurocidin, cathelicidin, and lysozyme. Further, several proteinases, such as neutrophil elastase and cathepsin G, contribute to bacterial killing by digestion of bacterial outer membrane proteins (10), surface appendages (11), and virulence factors (12). The same enzymes and agents that are secreted into the phagosome may also be released into the extracellular environment. Thus, extracellular mechanisms result in killing of invading microorganisms but the released destructive enzyme also damage contiguous cells and tissues (13).

Neutrophil dysfunction, *e.g.* abnormalities in adherence, chemotaxis, phagocytosis, superoxide production and bactericidal activity, are all associated with aggressive periodontitis (14), but it is also recognised that hyper-functional neutrophils may also lead to tissue injury contributing to the clinical signs of disease (15).

While subgingival bacteria are considered to constitute the primary initiating factor in the pathogenesis of periodontitis, the risk of developing periodontal disease differs between individuals, suggesting that host factors are involved in determining susceptibility to the disease (14). Several *in vitro* studies have demonstrated hyper-reactive neutrophils in

patients with chronic periodontitis (16, 17), which may lead to an excessive local release of ROS and proteolytic enzymes resulting in periodontal tissue destruction.

The aim of this *ex vivo* investigation was to compare peripheral blood PMN phagocytosis and killing of *P. gingivalis* and *A. actinomycetemcomitans*, as well as the release of ROS and human neutrophil elastase (HNE) in aggressive and chronic periodontitis patients. The null-hypothesis was that there would be no differences in PMN function between aggressive and chronic periodontitis patients.

Materials and methods

Subject recruitment

Twelve patients with chronic and six with aggressive periodontitis were recruited from patients of the Department of Conservative Dentistry (Section of Periodontology), University of Jena.

The definition of chronic and aggressive periodontitis was based on the classification system of the 1999 International Workshop for a Classification System of Periodontal diseases and Conditions (18).

Patients with generalized chronic periodontitis were recruited when they demonstrated:

- attachment loss ≥ 5 mm at more than 30% of sites
- age ≥ 35 years

Patients with aggressive periodontitis were required to fulfil the following criteria:

- radiographic bone loss ≥ 50 % at a minimum of two different teeth
- ≥ 5 mm attachment loss on at least 3 different teeth (other than first molars or incisors)
- age ≥ 35 years at onset of disease

Periodontally-healthy subjects (n = 12) with no evidence of periodontal disease (all probing depths ≤ 3 mm and no attachment loss) were recruited as controls.

Subjects with significant systemic disease (e.g. diabetes mellitus, cancer or coronary heart disease), antibiotic therapy within the last 6 months and pregnant or lactating females were excluded. Further exclusion criteria were vitamin supplementation within the previous 3 months, and any special dietary requirements. Only non-smokers with no history of smoking were included in the study.

Ethical approval was obtained from the local ethics committee of the University of Jena. Written informed consent was obtained from each subject prior to participation.

Clinical assessments

Probing depths (PD) were measured with a periodontal probe (PCP-UNC 15, Hu Friedy, Leimen, Germany) at six sites per tooth. Bleeding on probing (BoP) was calculated as the percentage of positive sites per subject.

Sample collection

Fasting venous blood samples were collected from the antecubital fossa (lithium heparin tube, Monovette, Sarstedt AG, Nümbrecht, Germany) from patients and controls between 8.00 and 10.00 a.m. after an overnight fast. From all subjects PMNs were isolated using dextran sedimentation followed by hypotonic lysis of erythrocytes. PMNs were resuspended with Hanks balanced salt solution (HBSS) to a density of 10^7 /ml. The number and viability of the neutrophils isolated was then determined by Trypan blue staining. At all times, viability exceeded 95%.

Bacterial strains

P. gingivalis ATCC 33277 and *A. actinomycetemcomitans* Y4 were obtained from the German strain collection DSMZ, Braunschweig, Germany. The strains were subcultivated on Schaedler's agar enriched with 10 % sheep blood and vitamin K for 16 h in the appropriate atmosphere, harvested and resuspended in 2.5 ml phosphate buffered saline (PBS) to a number of 10^8 bacteria/ml. To opsonize bacteria, after repeated washing with PBS, 200 μ l of the patient's own serum was mixed with 200 μ l PBS and added to the suspended cells for 10 min in an atmosphere with 5% CO₂ at 37°C. The suspension was then centrifuged again and 2.5 ml Hanks HBSS added.

Phagocytosis

PMN and bacterial suspensions were mixed at a 1:1 to results in a cellular ratio of PMNs to bacteria of 1:20. The mixture was then incubated for up to 2 h in a CO₂ atmosphere at 37°C. At different time points (0 min, 30 min, 60 min, 120 min), the suspension was centrifuged at 400 g for 4 min at 20°C. From the resultant supernatants, aliquots were dispensed onto Schaedler's agar plates to enumerate the colony forming units (CFUs) as the number of viable extracellular bacteria. The remaining supernatant was kept at -20° for further analysis of HNE activity. The spun cells were washed twice with PBS and then lysed by the addition of icecold water for 15 min. The lysate was also plated to determine the number (CFUs) of intracellular (i.e. phagocytosed but not killed) bacteria. Controls of bacterial suspensions mixed 1:1 with HBSS were stored under the same conditions to ensure that bacterial survival could be expected for at least the duration of the experiment.

Detection of immunoglobulin (Ig) G to *P. gingivalis* and *A. actinomycetemcomitans*

Whole bacterial cells of *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4 were used as antigens in the solid-phase ELISA. Bacteria were subcultured in Schaedler broth enriched with 1 μ g/ml vitamin K for three days. The cultures were checked for purity by culture and pelleted by centrifugation at 5000 g for 15 min. The pellets were washed three times with PBS, resuspended in PBS supplemented with 0.1 sodium azide, treated by sonication and stored at 4°. The inactivation was checked by culture.

The bacterial suspensions were diluted to 10 µg / ml protein. Each 100 µl of bacterial suspension was given to a well of 96 well MaxiSorp™-microtiter-plate (NUNC A/S, Roskilde, Denmark). The plates were kept at 4°C overnight. After removing the suspension 200 µl of blocking solution (4% bovine serum albumine (BSA) (SERVA, Heidelberg, Germany) in PBS) was added for 2 h. The plates were washed three times with PBS supplemented with 0.005% Tween 20. Serum samples diluted 1 : 50 with PBS containing 0.4% BSA were placed into the plates. After 1 h of incubation and a washing as described before, HRP-labelled mouse anti-human IgG (Dako, Glostrup, Denmark) were added. The plates were then incubated again for 1 h before washing as before. As substrate 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich, Munich, Germany) was used. After stopping the reaction by addition of 2 N sulphuric acid, the plates were read in a spectrophotometer at 450 nm.

Human neutrophil granulocyte elastase activity

HNE activity of the supernatants was measured with a microplate assay by using the chromogenic substrate N-Methoxysuccinyl-Ala-Ala-pro-Val-pNa (Sigma-Aldrich, Taufkirchen, Germany) (19). The substrate was dissolved in dimethylsulfoxide (DMSO) to 10 mM, and the working solution was made up to 1 mM by dilution with 0.05 M Tris, pH 7.5. In brief, to each 90 µl aliquot of the eluate of the specimen, 10 µl of the substrate working solution was added. Absorbency at 405 nm was measured immediately and also after incubation at 37°C for 30 minutes in a microplate reader. One unit (U) was calculated as the amount of enzyme that hydrolyzed 1 nmol of substrate in 1 minute.

Release of oxygen radicals

The total release of oxygen radicals was measured with luminol-enhanced chemiluminescence and the extracellular release of oxygen radicals with isoluminol-enhanced chemoluminescence (20).

Bacterial and PMN suspensions were prepared as described above. 100 µl aliquots were mixed in a well of a microtiter-plate with addition of 50 µl luminol or isoluminol (3.0 mM). The maximal light intensity was recorded in a luminometer (Applied Biosystems, Foster City, CA, USA) up to 2 h. PMA (phorbol-12 myristate-13 acetate) was used as a positive control, and HBSS constituted the negative control.

Statistical analysis

The clinical data and the levels of the observed parameters were expressed as means ± standard deviation (SD). The significance of differences between groups was assessed using ANOVA with post-hoc Bonferroni-tests. A p-value of < 0.05 was considered to be statistically significant. SPSS 13.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

Demographic and clinical data are presented in Table 1. Patients with periodontal disease had significantly higher mean probing depths and BOP scores than healthy controls

($p < 0.05$). No differences in clinical parameters were detected between aggressive and chronic periodontitis.

Phagocytosis and killing of bacteria

At the first measurement after 30 minutes PMNs from patients with chronic periodontitis ($62.16\% \pm 19.39\%$) and aggressive periodontitis ($43.26\% \pm 26.63\%$) phagocytosed more *P. gingivalis* ATCC 33277 than those from the healthy controls ($24.43\% \pm 19.87\%$). The difference between chronic periodontitis and the controls was statistically significant ($p < 0.05$). From 30 minutes to 60 minutes after bacterial exposure, phagocytic activity increased in all groups. At this time-point, the phagocytic activity in the control group was only about one third that observed in the periodontitis groups ($p < 0.05$). After this however, a significant increase in phagocytic activity was recorded in the control group from 60 to 120 minutes. The phagocytic activity of PMNs in chronic periodontitis ($52.57\% \pm 23.77\%$) decreased from 60 to 120 minutes while in aggressive periodontitis, phagocytosis ($81.88 \pm 33.97\%$) demonstrated a further increase. The percentage of the killed bacteria was appropriate when considering the corresponding phagocytic activity. The ratio of killed to phagocytosed bacteria was comparable in all groups (Figure 1). Only PMNs of subjects with chronic periodontitis killed more phagocytosed bacteria than PMNs from aggressive periodontitis patients and controls at the baseline examination (30 minutes after start of phagocytosis).

After 30 minutes, *A. actinomycetemcomitans* Y4 was phagocytosed by PMNs to a greater degree in chronic periodontitis ($53.21\% \pm 24.29\%$) and aggressive periodontitis ($73.10\% \pm 13.64\%$) than from PMNs of healthy controls ($34.20\% \pm 23.05\%$). The difference between aggressive periodontitis PMNs and those of the control group was significant ($p < 0.05$). Phagocytic activity decreased in the chronic periodontitis group (from $58.57\% \pm 19.32\%$ to $47.56\% \pm 23.73\%$) and in the control group (from $47.68\% \pm 14.87\%$ to $34.53\% \pm 25.01\%$) from 60 minutes to 120 minutes whereas phagocytosis increased in PMNs from subjects with aggressive periodontitis ($64.37\% \pm 12.07\%$ to $83.05\% \pm 9.19\%$) over the same time period. The differences after 120 minutes between aggressive and chronic periodontitis as well as controls were significant ($p < 0.05$). The percentages of killed bacteria were equivalent to the percentages of phagocytosed bacteria. Results are illustrated in Figure 2.

Anti-*Porphyromonas gingivalis*-IgG and anti-*Aggregatibacter actinomycetemcomitans*-IgG

Antibodies to *P. gingivalis* and *A. actinomycetemcomitans* were detected in serum of patients and controls (Figure 3). Patients with aggressive and chronic periodontitis had significantly higher anti-*P. gingivalis*-titres than the healthy controls ($p < 0.05$). Significantly elevated IgG titres in aggressive periodontitis were measured to *A. actinomycetemcomitans* ($p < 0.05$).

Chemiluminescence

The results of total and extracellular ROS release are presented in Figure 4. The luminal-dependent chemiluminescence suggests that PMNs from subjects with chronic and aggressive periodontitis released significantly more ROS in the absence of any stimulus than PMNs from healthy donors ($p < 0.05$). The total release of ROS increased in chronic

periodontitis and in the control group after interaction with *P. gingivalis* ATCC 33277. The interaction with *A. actinomycetemcomitans* Y4 led to more total ROS release in chronic periodontitis and in the control group than that observed after exposure to *P. gingivalis* ATCC 33277. For both bacterial strains, luminol-dependent chemiluminescence was significantly less in aggressive periodontitis than in chronic periodontitis and in the control group ($p < 0.05$).

The isoluminol-dependent chemiluminescence measured the extracellular release of reactive oxygen species. PMNs in chronic and aggressive periodontitis were probably hyper-active, because they released significant more ROS than the PMNs from the controls ($p < 0.05$) in an unstimulated state. PMNs in chronic periodontitis also seemed to be hyper-reactive in interaction with *A. actinomycetemcomitans* Y4, as they released significantly more ROS than PMNs from subjects with aggressive periodontitis or healthy controls ($p < 0.05$). The extracellular release of reactive oxygen species from PMNs following interaction with *P. gingivalis* ATCC 33277 was (not significantly) lower in aggressive periodontitis patients compared to chronic periodontitis patients and periodontally healthy subjects.

Elastase activity

Immediately after isolation, and without any additional stimulation, extracellular HNE activity was significantly lower in periodontally healthy controls than in chronic and aggressive periodontitis patients (each $p < 0.05$). However, after interaction with both bacterial species the only significant difference was a decreased HNE activity in PMNs of chronic periodontitis patients compared to controls 120 min after exposure to *P. gingivalis* ATCC 33277 (Table 2).

Discussion

This *in vitro* study investigated the ability of peripheral PMNs of subjects with periodontal diseases to phagocytose and kill *P. gingivalis* and *A. actinomycetemcomitans*. Broadly speaking, we identified that PMNs from aggressive periodontitis patients demonstrated enhanced phagocytosis activities and released less ROS release than PMNs from chronic periodontitis patients.

PMNs, essential for innate host defences against invading microorganisms, eliminate pathogens by phagocytosis. During phagocytosis, PMNs produce ROS and release cytotoxic granule components into pathogen-containing phagocytic vacuoles to kill the bacteria (20). PMNs collected from crevicular fluid have been shown to have defective phagocytosis in localized aggressive periodontitis (21) and in generalized aggressive periodontitis (22).

Our phagocytosis assay was adapted from the method described by Hampton *et al.* (23). This method determines extracellular free floating viable bacteria as well as phagocytosed bacteria. One limitation of the method is the inability to differentiate between adherent and ingested (intracellular) viable bacteria. Furthermore, it is not possible to distinguish between intracellular and extracellular killed bacteria. Notwithstanding these limitations, the phagocytic activity of blood PMNs from subjects with aggressive periodontitis was different to that of PMNs from chronic periodontitis patients or healthy controls. Indeed, we observed

an ongoing increase of phagocytosis of *A. actinomycetemcomitans* and *P. gingivalis* by PMNs from patients with aggressive periodontitis. In chronic periodontitis, more phagocytosis of *P. gingivalis* was identified. In our assay, the patients' own serum was used for opsonisation. The patients are infected by these periopathogenes and might therefore have high levels of specific antibodies against these species. High antibody levels against *A. actinomycetemcomitans* were detected in subjects with localized aggressive periodontitis (1) and against *P. gingivalis* in adults with severe periodontal destruction (24). Several studies reported that in periodontitis an increased systemic IgG level can be observed (25–27). Gibson III *et al.* (28) reported that specific IgG against components of *P. gingivalis* in the sera of patients promotes the uptake of that species. IgG antibodies may enhance phagocytosis and killing of oral microorganisms through activation of complement and opsonization (29, 30). This might explain the higher phagocytosis activity of PMNs in periodontitis patients at the first observation (30 min). Local infections, including periodontal disease, cause variations of activating factors in plasma and lead to intravascular PMN priming (31). Nevertheless, leukotoxin, an important virulence factor of *A. actinomycetemcomitans* exhibits cytolytic activity against PMNs and macrophages (32).

In our study the bacteria are killed to a high extent, indicating a robust PMN response. Neutrophil-mediated bacterial killing involves both oxygen-independent and oxygen-dependent processes (6). Granule fractions consisting of elastase, azurocidin and cathepsin G are bactericidal against *A. actinomycetemcomitans*, though strain-dependent differences in bactericidal activity do exist (33). The ratio of killed to phagocytosed bacteria was similar for *A. actinomycetemcomitans* Y4 in all three groups, indicating that nearly all bacteria which were phagocytosed were also killed by the neutrophils. Only the PMNs from patients with chronic periodontitis killed more phagocytosed *P. gingivalis* ATCC 33277 than the PMNs of healthy controls or patients with aggressive periodontitis at the 30 minutes observation (each $p < 0.05$). However, it is also important to remember that bacterial killing by PMNs also occurs extracellularly by the formation of extracellular traps (34).

During interactions with plaque bacteria, PMNs cause tissue destruction via extracellular release of lysosomal enzymes, granule components and oxygen radicals (35). Elastase is one of the proteolytic enzymes present in the PMN primary granules, released on activation of the PMN and capable of degrading extracellular matrix proteins of the connective tissue (36). Higher granulocyte elastase activity has previously been observed in patients with periodontitis (both aggressive and chronic) in comparison to healthy controls (37) and our observations confirm these findings in that PMNs from aggressive and chronic periodontitis patients released significantly more elastase even in absence of any stimulation. Increased release of ROS and elastase by PMNs is likely to contribute to enhanced tissue destruction in periodontal disease (38).

The extracellular release of ROS can cause lipid peroxidation (39) and may also result in damage to the surrounding tissues as well (40). Sheiki *et al.* (2001) suggested that periodontal pathogens might stimulate PMNs to release ROS, and in their *in-vitro* study, *Fusobacterium nucleatum* was able to stimulate PMNs to produce ROS (41). The intra- and extracellular release of oxygen radicals after phagocytosis of *P. gingivalis* and *A. actinomycetemcomitans* was investigated in another *in vitro* study and these data suggest

that after phagocytosis of *P. gingivalis*, superoxide was produced to a lesser extent compared to that seen after phagocytosis of *A. actinomycetemcomitans* (40). Our data confirm this observation in the context of CP patients. Here, we measured less total and extracellular ROS release (luminal-dependent and isoluminol-dependent chemiluminescence) when PMNs phagocytosed *P. gingivalis* ATCC 33277 in comparison to *A. actinomycetemcomitans* Y4.. Nevertheless it should be mentioned, that strain dependent differences exist among *A. actinomycetemcomitans* strains in their ability to trigger the luminal amplified chemiluminescence (41).

Interestingly, the release of ROS in the absence of any stimulation with both tested bacteria was higher in PMNs from chronic and aggressive periodontitis patients than in the periodontally healthy control group. This is supported by recent research by Matthews et al. (2007) who reported that PMNs in chronic periodontitis were hyperactive in terms of extracellular release of ROS without exogenous stimulation (42). In a further analysis they demonstrated that this release of ROS in the absence of stimulation was also detectable after periodontal therapy (43). These findings indicate that a baseline, unstimulated hyperactivity is constitutive in nature and could represent a risk factor for the development and progression of periodontitis (42, 43). Furthermore, genotyping studies suggested that the disposition for hyperactive PMNs may be a genetic risk factor for increased periodontal destruction (36).

While PMNs from subjects with chronic periodontitis and from the controls could be stimulated by *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4, the PMNs from aggressive periodontitis patients did not release additional ROS after contact with those species. This observed lower ROS release in aggressive periodontitis after contact with periodontal pathogens is in contrast to data reported by Shapira *et al.* (1991) who identified PMN hyper-activity PMNs after stimulation with opsonized bacteria in subjects with rapidly progressive periodontitis (now renamed as generalized aggressive periodontitis) (44). However, the difference between this study and our own may result from the different methods that were employed; we investigated the direct interaction between PMNs and periodontal pathogens while Shapira and co-worker (44) used streptococci opsonized with histone.

Conclusions

Within the limitations of our study, the results indicate that PMNs in aggressive and chronic periodontitis are hyperactive even without any particular stimulus. Furthermore, periodontal bacteria that were opsonized by the patients' own serum were efficiently phagocytosed and killed by PMNs. An extracellular release of ROS and neutrophil elastase by PMNs was detected and likely not only kill bacteria extracellularly but also damage the surrounding periodontal tissues.

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Figure 1.

Phagocytosis and killing of *P. gingivalis*. PMNs from patients with chronic periodontitis phagocytosed significantly more *P. gingivalis* at the 30 min and 60 min time point than PMNs from controls (* p<0.05) (A). The percentage of killed bacteria followed a similar pattern (B). PMNs in chronic periodontitis killed more of the phagocytosed bacteria than PMNs from subjects with aggressive periodontitis or controls at 30 and 120 min (C).

Figure 2.

Phagocytosis and Killing of *Aggregatibacter actinomycetemcomitans*. PMNs from aggressive periodontitis patients phagocytosed more *A. actinomycetemcomitans* than PMNs from controls at the 30 min and 120 min observations (* $p < 0.05$) and more than PMNs of patients with chronic periodontitis after 120 minutes (A). The killing of the phagocytosed bacteria correlated with this observation (B). Nearly all phagocytosed bacteria were also killed by the PMNs, as indicated by the high killed/phagocytosed ratios (C).

A

B

Figure 3.

Systemic antibodies to *P. gingivalis* (A) and *A. actinomycetemcomitans* (B) in aggressive and chronic periodontitis in comparison to periodontally healthy subjects. Patients with periodontitis had significantly higher titres of anti-*P. gingivalis* IgG than the controls (A). Only in subjects with aggressive periodontitis was the IgG titre for anti-*A. actinomycetemcomitans* significantly higher than in the periodontally-healthy controls (B).

Figure 4.

Total (A) and extracellular (B) release of reactive oxygen species (ROS) by peripheral blood PMNs from chronic and aggressive periodontitis patients in comparison to healthy controls determined by chemoluminescence (counts). (A) PMNs in chronic and aggressive periodontitis release significantly more ROS than PMNs from controls in the absence of stimulation (* $p < 0.05$). PMNs in aggressive periodontitis release significantly less ROS than PMNs from subjects with chronic periodontitis (#) and controls (*) after stimulation with *P. gingivalis* (*P.g.*) and *A. actinomycetemcomitans* (*A.a.*).

(B) The extracellular release of ROS by PMNs from patients with aggressive periodontitis was also lower than that from PMNs of subjects with chronic periodontitis (# $p<0.05$). PMNs in chronic periodontitis released significantly more ROS after interaction with *A. actinomycetemcomitans* than PMNs from the controls (* $p<0.05$).

Table 1

Demographic and clinical data

	Control n=12	Chronic periodontitis n=12	Aggressive periodontitis n=6
Age (mean $\pm$ SD) (years)	38.1 $\pm$ 10.7	52.3 $\pm$ 9.4 ^{*#}	35.5 $\pm$ 6.9
Gender (m:f)	6:6	7:5	3:3
Baseline PD (mean $\pm$ SD) (mm)	1.58 $\pm$ 0.30	5.31 $\pm$ 0.76 [*]	5.67 $\pm$ 0.59 [*]
Baseline BoP (mean $\pm$ SD) (%)	6.98 $\pm$ 8.78	80.76 $\pm$ 22.28 [*]	79.84 $\pm$ 19.76 [*]

^{*} Significantly different from control group (p < 0.05)

[#] Significantly different from aggressive periodontitis (p < 0.05)

Table 2

Human neutrophil elastase activity (mU) of PMNs from subjects with aggressive and chronic periodontitis in comparison to periodontally healthy controls without stimulus and after contact with *P. gingivalis* and *A. actinomycetemcomitans*. ($p < 0.05$ in comparison with healthy controls at that time point)

HNE activity (mU)	Aggressive periodontitis	Chronic periodontitis	Healthy controls
Without stimulus			
0 min	81.25 ± 38.54*	64.75 ± 48.09*	8.00 ± 4.03
30 min	60.50 ± 51.20	39.00 ± 35.60	8.75 ± 7.54
60 min	26.50 ± 14.65	4.50 ± 4.00	1.00 ± 0.75
120 min	39.00 ± 32.33	2.33 ± 0.58	2.50 ± 1.36
With <i>P. gingivalis</i> ATCC 33277			
30 min	24.86 ± 16.72	12.50 ± 3.73	4.20 ± 3.71
60 min	9.14 ± 4.92	3.50 ± 2.56	5.80 ± 4.18
120 min	16.71 ± 4.51	2.60 ± 1.67*	4.60 ± 2.41
With <i>A. actinomycetem-comitans</i> Y4			
30 min	6.00 ± 5.61	3.00 ± 2.74	6.29 ± 5.69
60 min	3.40 ± 2.13	2.40 ± 1.58	6.57 ± 4.21
120 min	4.60 ± 4.45	0.75 ± 0.62	6.43 ± 3.83