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

A. Guentsch[1], M. Puklo[2], P. M. Preshaw[3], E. Glockmann[1], W. Pfister[4], J. Potempa[2,5], S. Eick[4]

[1]Department of Conservative Dentistry, University Hospital, Friedrich-Schiller-University Jena, Jena, Germany, [2]Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland, [3]Department of Periodontology, School of Dental Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne, UK, [4]Institute of Medical Microbiology, University Hospital, Friedrich-Schiller-University Jena, Jena, Germany and [5]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA, USA

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Background and Objective: This study analyzed the interaction of *Porphyromonas gingivalis* ATCC 33277 and *Aggregatibacter actinomycetemcomitans* Y4 with peripheral blood polymorphonuclear neutrophils taken from patients with aggressive periodontitis and chronic periodontitis.

Material and Methods: Peripheral blood polymorphonuclear neutrophils obtained from 12 patients with chronic periodontitis, six patients with aggressive periodontitis and 12 healthy controls were exposed to *P. gingivalis* and *A. actinomycetemcomitans* following opsonization of the bacteria using the patient's own serum. Serum immunoglobulin G (IgG) levels against both periodontopathogens were measured. Phagocytosis and killing of the bacteria, as well as the extracellular human neutrophil elastase activity, were quantified. The total amount and the extracellular release of reactive oxygen species were measured using luminol-dependent and isoluminol-dependent chemiluminescence.

Results: Polymorphonuclear neutrophils from patients with chronic (62.16 ± 19.39%) and aggressive (43.26 ± 26.63%) periodontitis phagocytosed more *P. gingivalis* than the healthy controls (24.43 ± 19.87%) at the 30-min time point after exposure to the bacteria ($p < 0.05$). High serum IgG levels against *P. gingivalis* and *A. actinomycetemcomitans* were detected in subjects with periodontitis. Polymorphonuclear neutrophils from subjects with chronic and aggressive periodontitis released significantly more reactive oxygen species and demonstrated greater human neutrophil elastase activity in the absence of any stimulus than polymorphonuclear neutrophils from healthy controls ($p < 0.05$). Polymorphonuclear neutrophils in chronic periodontitis released significantly more reactive oxygen species when exposed to *P. gingivalis* and *A. actinomycetemcomitans* than polymorphonuclear neutrophils in aggressive periodontitis.

Conclusion: High serum IgG levels against *P. gingivalis* and *A. actinomycetemcomitans* promote phagocytosis in periodontitis. The extracellular release of

Arndt Guentsch, Department of Conservative Dentistry, University Hospital, Friedrich-Schiller-University Jena, An der alten Post 4, D-07743 Jena, Germany
Tel: +49 3641 934409
Fax: +49 3641 934411
e-mail: arndt.guentsch@med.uni-jena.de

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reactive oxygen species and neutrophil elastase by polymorphonuclear neutrophils may also contribute to damage of the surrounding periodontal tissues.

Periodontitis is a chronic inflammation of the periodontal tissues that occurs 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 represent the first line of cellular host responses against bacteria in the gingival sulcus (5). The antimicrobial activities of polymorphonuclear neutrophils include oxygen-dependent and oxygen-independent mechanisms (6). The oxygen-dependent pathway involves the production of reactive oxygen species, molecules that are capable of initiating periodontal tissue destruction (7). The production of reactive oxygen species by polymorphonuclear neutrophils is primarily focused towards bacterial killing, but extracellular release of reactive oxygen species results in collateral damage of the surrounding tissues (8). Nonoxidative microbial killing relies on the contents of three cytoplasmic granule subsets: the azurophilic (primary) granules; specific (secondary) granules; 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. Furthermore, 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 the death of invading microorganisms but the released destructive enzymes also damage contiguous cells and tissues (13).

Neutrophil dysfunction (e.g. abnormalities in adherence, chemotaxis, phagocytosis, superoxide production and bactericidal activity) is associated with aggressive periodontitis (14), but it is also recognized that hyperfunctional neutrophils may 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 the presence of hyper-reactive neutrophils in patients with chronic periodontitis (16,17), which may lead to an excessive local release of reactive oxygen species and proteolytic enzymes, resulting in periodontal tissue destruction.

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

Material and methods

Subject recruitment

Twelve patients with chronic periodontitis and six patients 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 the following (19).

(i) Attachment loss ≥ 5 mm at more than 30% of sites.
(ii) Age ≥ 35 years.

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

(i) Radiographic bone loss ≥ 50% at a minimum of two different teeth.
(ii) ≥ 5 mm attachment loss on at least three different teeth (other than first molars or incisors).
(iii) 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), those who had received antibiotic therapy within the last 6 mo and pregnant or lactating women were excluded. Further exclusion criteria were vitamin supplementation within the previous 3 mo and any special dietary requirements. Only nonsmokers 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 were measured using a periodontal probe (PCP-UNC 15; Hu Friedy, Leimen, Germany) at six sites

per tooth. Bleeding on probing was calculated as the percentage of positive sites per subject.

Sample collection

Fasting venous blood samples were collected from the antecubital fossa (into a lithium heparin tube, Monovette; Sarstedt AG, Nümbrecht, Germany) from patients and controls between 08:00 and 10:00 h after an overnight fast. Polymorphonuclear neutrophils were isolated from all subjects using dextran sedimentation followed by hypotonic lysis of erythrocytes. Polymorphonuclear neutrophils were resuspended in Hanks' balanced salt solution to a density of 10^7 cells/mL. The number and viability of the neutrophils isolated was then determined by Trypan Blue staining. At all times, viability exceeded 95%.

Bacterial strains

Porphyromonas gingivalis ATCC 33277 and *A. actinomycetemcomitans* Y4 were obtained from the German strain collection DSMZ, Braunschweig, Germany. The strains were subcultured on Schaedler's agar enriched with 10% sheep blood and vitamin K for 16 h in an anaerobic (*P. gingivalis*) and with 5% CO_2 (*A. actinomycetemcomita*) atmosphere, respectively, then harvested and resuspended in 2.5 mL of phosphate-buffered saline to a concentration of 10^8 bacteria/mL. To opsonize bacteria, after repeated washing with phosphate-buffered saline, 200 μL of the patient's own serum was mixed with 200 μL of phosphate-buffered saline and added to the suspended cells for 10 min in an atmosphere of 5% CO_2 at 37°C. The suspension was then centrifuged again and 2.5 mL of Hanks' balanced salt solution was added.

Phagocytosis

Polymorphonuclear neutrophil and bacterial suspensions were mixed at a ratio of 1:1 to give a cellular ratio of polymorphonuclear neutrophils to bacteria of 1:20. The mixture was then incubated for up to 2 h in an atmosphere of 5% CO_2 at 37°C. At different time points (0, 30, 60 and 120 min), the suspension was centrifuged (400 *g*, 4 min, 20°C). From the resultant supernatants, aliquots were dispensed onto Schaedler's agar plates to enumerate the colony-forming units as the number of viable extracellular bacteria. The remaining supernatant was kept at −20°C for further analysis of human neutrophil elastase activity. The spun cells were washed twice with phosphate-buffered saline and then lysed by the addition of ice-cold water for 15 min. The lysate was also plated to determine the number (colony-forming units) of intracellular (i.e. phagocytosed but not killed) bacteria.

Controls of bacterial suspensions mixed 1:1 with Hanks' balanced salt solution were stored under the same conditions to ensure that bacterial survival could be expected for at least the duration of the experiment. The viability of the bacteria (baseline 5×10^7 bacteria/mL) remained constant over the observation period (range ± 10%). Furthermore, as controls, polymorphonuclear neutrophils were prepared in the same way and mixed 1:1 with Hanks' balanced salt solution without any bacteria.

Detection of immunoglobulin 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 enzyme-linked immunosorbent assay. Bacteria were subcultured for 3 d in Schaedler broth enriched with 1 μg/mL of vitamin K. The cultures were checked for purity by culturing a sample of bacterial suspension on solid agar and pelleted by centrifugation at 5000 *g* for 15 min at 20°C. The pellets were washed three times with phosphate-buffered saline, resuspended in phosphate-buffered saline supplemented with 0.1% sodium azide, treated for 1 min by sonication (35 kHz, 120 W) and stored at 4°C. The killing of bacteria was checked by culture.

The bacterial suspensions were diluted to 10 μg/mL of protein. Then, 100 μL of bacterial suspension was placed in each well of a 96-well MaxiSorp™ microtiter plate (NUNC A/S, Roskilde, Denmark). The plates were stored at 4°C overnight. After removing the suspension, 200 μL of blocking solution [4% bovine serum albumin (SERVA, Heidelberg, Germany) in phosphate-buffered saline] was added for 2 h. The plates were washed three times with phosphate-buffered saline supplemented with 0.005% Tween 20. First, wells treated with serial dilutions of a pooled serum sample were used to construct reference curves. Then, serum samples diluted 1:5000 and an additional two serial dilutions with phosphate-buffered saline containing 0.4% bovine serum albumin were added to the plates. After 1 h of incubation and washing as described above, horseradish peroxidase-labelled mouse anti-human IgG (Dako, Glostrup, Denmark) were added. The plates were then incubated again for 1 h before washing as described above. As substrate, 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, Munich, Germany) was used. After stopping the reaction by addition of 2 mol sulphuric acid, the plates were read in a spectrophotometer at 450 nm. The results were interpolated from the standard curve and expressed as arbitrary units based on a value of 1.000 for the pooled serum sample diluted 1:5000.

Human neutrophil granulocyte elastase activity

Human neutrophil elastase 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) (20). The substrate was dissolved in dimethylsulfoxide 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. Absorbance at 405 nm was measured immediately, and also after incubation at 37°C for 30 min, using a microplate

reader. One unit (U) was calculated as the amount of enzyme that hydrolyzed 1 nmol of substrate in 1 min.

Release of oxygen radicals

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

Bacterial and polymorphonuclear neutrophil suspensions were prepared as described above. Aliquots of 100 μL were mixed in a well of a microtiter plate after the addition of 50 μL of 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. Phorbol-12 myristate-13 acetate (PMA) was used as a positive control, and Hanks' balanced salt solution was used as the negative control.

Statistical analysis

The clinical data and the levels of the observed parameters were expressed as means ± standard deviation. The significance of differences between groups was assessed using analysis of variance 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 bleeding on probing scores than healthy controls ($p < 0.05$). No differences in clinical parameters were detected between patients with aggressive periodontitis and chronic periodontitis.

Phagocytosis and killing of bacteria

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

Table 1. Demographic and clinical data

	Control ($n = 12$)	Chronic periodontitis ($n = 12$)	Aggressive periodontitis ($n = 6$)
Age (mean ± SD) (years)	38.1 ± 10.7	52.3 ± 9.4*,**	35.5 ± 6.9
Gender (m : f)	6:6	7:5	3:3
Baseline PD (mean ± SD) (mm)	1.58 ± 0.30	5.31 ± 0.76*	5.67 ± 0.59*
Baseline BOP (mean ± SD) (%)	6.98 ± 8.78	80.76 ± 22.28*	79.84 ± 19.76*

*Significantly different from the control group ($p < 0.05$).
**Significantly different from the aggressive periodontitis group ($p < 0.05$).
BOP, bleeding on probing; f, female; m, male; PD, probing depth; SD, standard deviation.

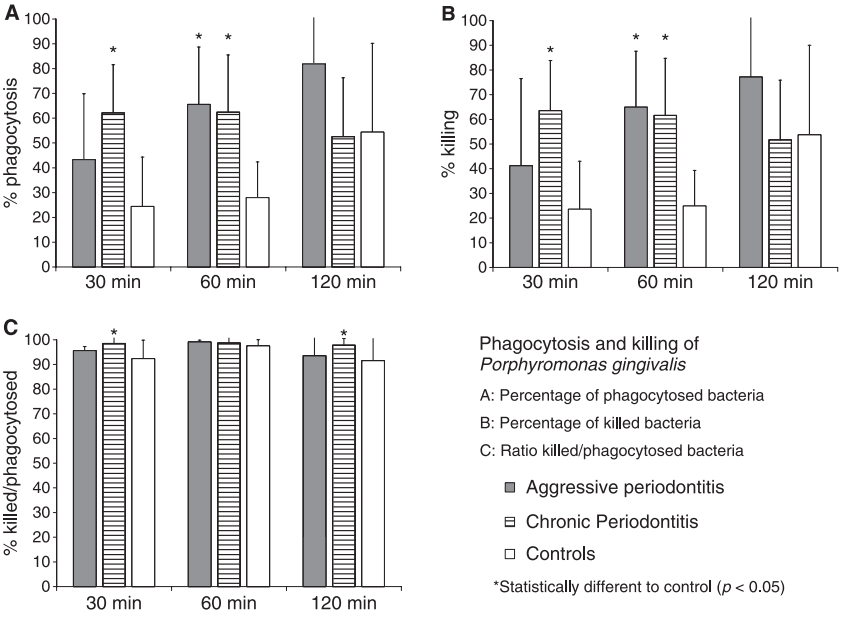

Fig. 1. Phagocytosis and killing of *Porphyromonas gingivalis*. (A) Polymorphonuclear neutrophils from patients with chronic periodontitis phagocytosed significantly more *P. gingivalis* at the 30-min and 60-min time points than polymorphonuclear neutrophils from controls (*$p < 0.05$). (B) The percentage of killed bacteria followed a similar pattern. (C) Polymorphonuclear neutrophils from patients with chronic periodontitis killed more of the phagocytosed bacteria than polymorphonuclear neutrophils from subjects with aggressive periodontitis or from controls at 30 and 120 min.

(30 min after the start of phagocytosis).

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

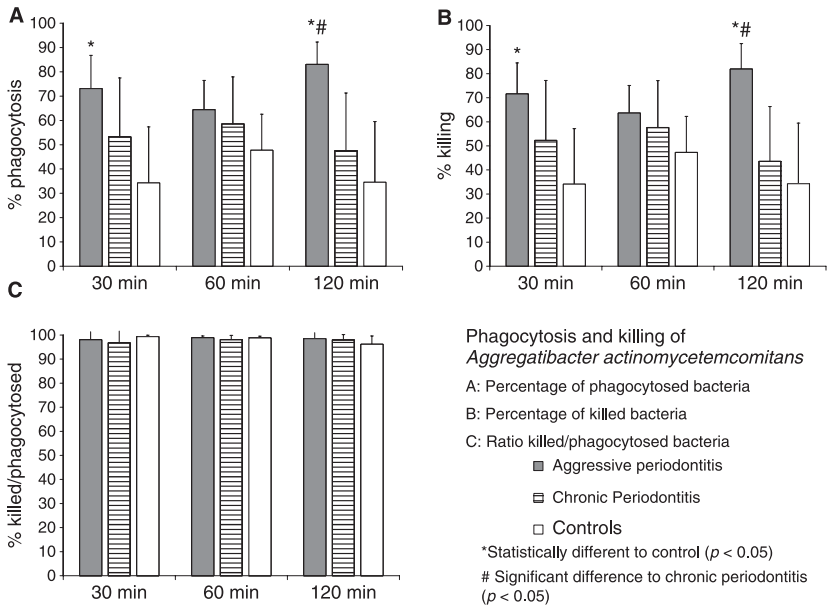

Fig. 2. Phagocytosis and killing of *Aggregatibacter actinomycetemcomitans*. (A) Polymorphonuclear neutrophils from patients with aggressive periodontitis phagocytosed more *A. actinomycetemcomitans* than polymorphonuclear neutrophils from controls at the 30-min and 120-min time points (*$p < 0.05$) and more than polymorphonuclear neutrophils of patients with chronic periodontitis after 120 min. (B) The killing of the phagocytosed bacteria correlated with this observation. (C) Nearly all phagocytosed bacteria were also killed by the polymorphonuclear neutrophils, as indicated by the high killed/phagocytosed ratios.

Anti-*P. gingivalis* IgG and anti-*A. actinomycetemcomitans* IgG

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

Chemiluminescence

The results of total and extracellular reactive oxygen species release are presented in Fig. 4. The luminol-dependent chemiluminescence suggests that polymorphonuclear neutrophils from subjects with chronic periodontitis and aggressive periodontitis released significantly more reactive oxygen species in the absence of any stimulus than polymorphonuclear neutrophils from healthy donors ($p < 0.05$). The total release of reactive oxygen species 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 reactive oxygen species 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 lower 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. Polymorphonuclear neutrophils in chronic and aggressive periodontitis were probably hyperactive because they released significantly more reactive oxygen species than the polymorphonuclear neutrophils from the controls ($p < 0.05$) in an unstimulated state. Polymorphonuclear neutrophils in chronic periodontitis also seemed to be hyper-reactive in interaction with *A. actinomycetemcomitans* Y4, as they released significantly more reactive oxygen species than polymorphonuclear neutrophils from subjects with aggressive periodontitis or healthy controls ($p < 0.05$). The extracellular release of reactive oxygen species from polymorphonuclear neutrophils following interaction with *P. gingivalis* ATCC 33277 was (not significantly) lower in aggressive periodontitis patients compared with chronic periodontitis patients and periodontally healthy subjects.

Elastase activity

Immediately after isolation, and without any additional stimulation, extracellular human neutrophil elastase activity was found to be significantly lower in periodontally healthy controls

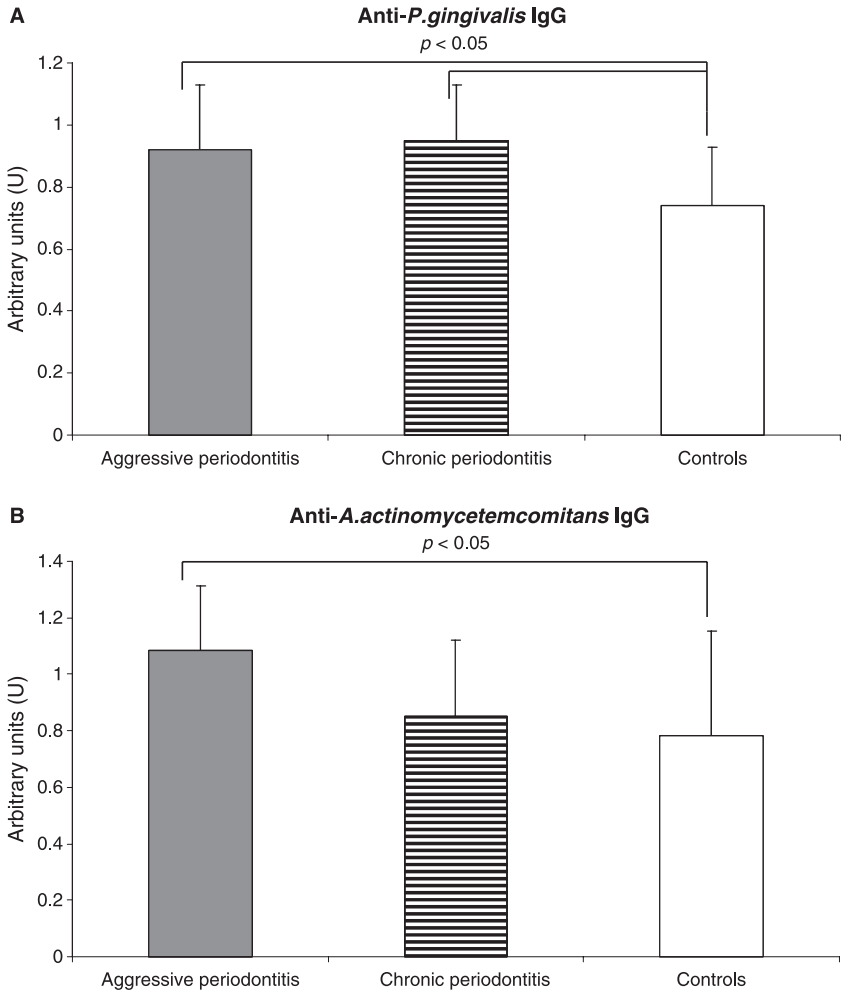

Fig. 3. Systemic antibodies to *Porphyromonas gingivalis* (A) and *Aggregatibacter actinomycetemcomitans* (B) in patients with aggressive and chronic periodontitis in comparison to periodontally healthy subjects. (A) Patients with periodontitis had significantly higher titres of anti-*P. gingivalis* immunoglobulin G (IgG) than the controls. (B) Only in subjects with aggressive periodontitis was the IgG titre for anti-*A. actinomycetemcomitans* significantly higher than in the periodontally healthy controls.

than in patients with chronic periodontitis or aggressive periodontitis (each $p < 0.05$). However, after exposure to *P. gingivalis* or *A. actinomycetemcomitans*, the only significant difference was a decreased human neutrophil elastase activity in polymorphonuclear neutrophils of chronic periodontitis patients compared with controls, 120 min after exposure to *P. gingivalis* ATCC 33277 (Table 2).

Discussion

This *ex vivo* study investigated the ability of peripheral polymorphonuclear neutrophils of subjects with periodontal diseases to phagocytose and kill *P. gingivalis* and *A. actinomycetemcomitans*. Broadly speaking, we identified that polymorphonuclear neutrophils from patients with aggressive periodontitis demonstrated enhanced phagocytosis activities and released less reactive oxygen species than polymorphonuclear neutrophils from patients with chronic periodontitis.

Polymorphonuclear neutrophils, essential for innate host defences against invading microorganisms, eliminate pathogens by phagocytosis. During phagocytosis, polymorphonuclear neutrophils produce reactive oxygen species and release cytotoxic granule components into pathogen-containing phagocytic vacuoles to kill the bacteria (21). Polymorphonuclear neutrophils collected from crevicular fluid have been shown to have defective phagocytosis in localized aggressive periodontitis (22) and in generalized aggressive periodontitis (23).

Our phagocytosis assay was adapted from the method described by Hampton *et al.* (24). This method identifies 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 polymorphonuclear neutrophils from subjects with aggressive periodontitis was different from that of polymorphonuclear neutrophils from patients with chronic periodontitis or healthy controls. Indeed, we observed an ongoing increase of phagocytosis of *A. actinomycetemcomitans* and *P. gingivalis* by polymorphonuclear neutrophils 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 opsonization. The patients are infected with these periopathogens 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 (25). Several studies have reported that in periodontitis an increased systemic IgG level can be observed (26–28). Gibson *et al.* (29) reported that specific IgG against components of *P. gingivalis* in the sera of patients promotes the uptake of that species. IgG may enhance the phagocytosis and killing of oral microorganisms through activation of complement and opsonization (30,31). This might explain the higher phagocytotic activity of polymorphonuclear neutrophils in periodontitis patients at

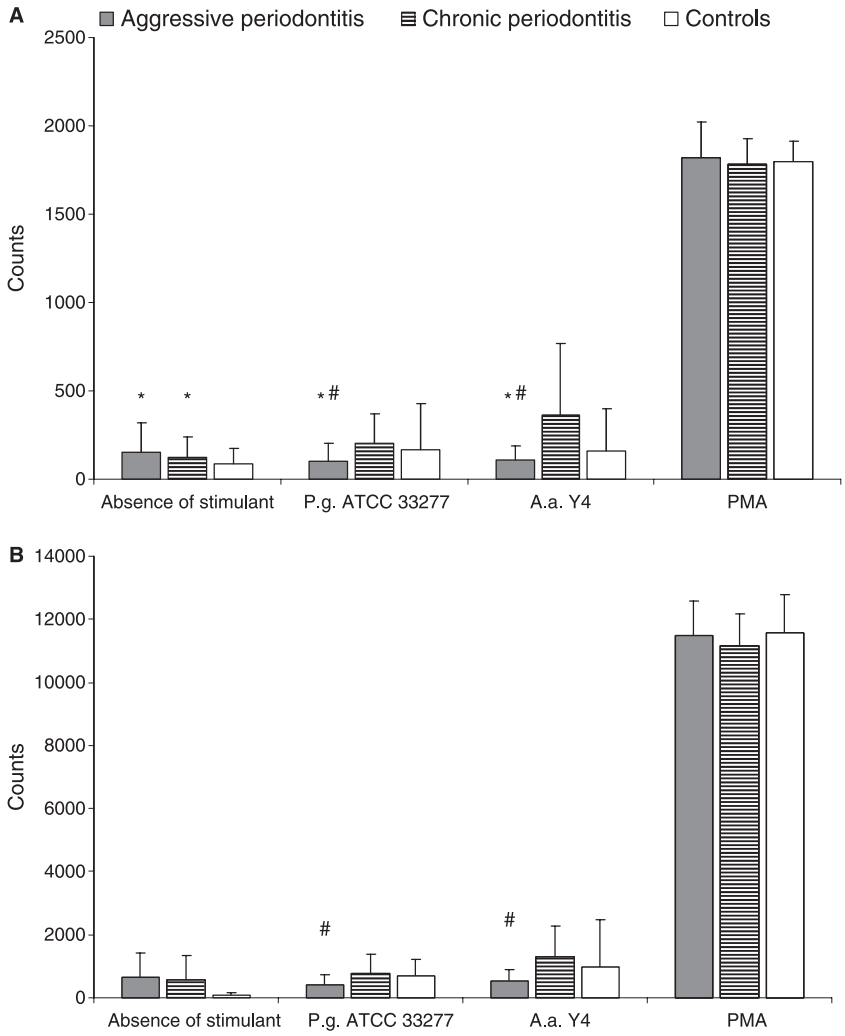

Fig. 4. Total (A) and extracellular (B) release of reactive oxygen species by peripheral blood polymorphonuclear neutrophils from patients with chronic and aggressive periodontitis in comparison to healthy controls as determined by chemoluminescence (counts). (A) Polymorphonuclear neutrophils from patients with chronic and aggressive periodontitis release significantly more reactive oxygen species than polymorphonuclear neutrophils from controls in the absence of stimulation (*$p < 0.05$). Polymorphonuclear neutrophils from patients with aggressive periodontitis release significantly less reactive oxygen species than polymorphonuclear neutrophils from subjects with chronic periodontitis () and controls (*) after stimulation with *Porphyromonas gingivalis* (*P. g.*) and *Aggregatibacter actinomycetemcomitans* (*A. a.*). (B) The extracellular release of reactive oxygen species by polymorphonuclear neutrophils from patients with aggressive periodontitis was also lower than that from polymorphonuclear neutrophils of subjects with chronic periodontitis (#$p < 0.05$). Polymorphonuclear neutrophils from patients with chronic periodontitis released significantly more reactive oxygen species after interaction with *A. actinomycetemcomitans* than polymorphonuclear neutrophils from the controls (*$p < 0.05$).

the first time point (30 min). Local infections, including periodontal disease, cause variations of activating factors in plasma and lead to intravascular polymorphonuclear neutrophil priming (32). Nevertheless, leukotoxin, an important virulence factor of *A. actinomycetemcomitans*, exhibits cytolytic activity against polymorphonuclear neutrophils and macrophages (33). The opsonization and phagocytosis of *P. gingivalis* depends on the presence of specific IgG (34), and the opsonization proceeds, in part, through the antibody-dependent alternate pathway of complement activation (35). Gingipains from *P. gingivalis* may also induce the production of proinflammatory cytokines and further modulate the host defenses (36).

In our study the bacteria were killed to a high extent, indicating a robust polymorphonuclear neutrophil 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*, although strain-dependent differences in bactericidal activity do exist (37). 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 polymorphonuclear neutrophils from patients with chronic periodontitis killed more phagocytosed *P. gingivalis* ATCC 33277 than the polymorphonuclear neutrophils of healthy controls or patients with aggressive periodontitis at the 30 min time point (each $p < 0.05$). However, it is also important to remember that bacterial killing by polymorphonuclear neutrophils also occurs extracellularly by the formation of extracellular traps (38).

During interactions with plaque bacteria, polymorphonuclear neutrophils cause tissue destruction via the extracellular release of lysosomal enzymes, granule components and oxygen radicals (39). Elastase is one of the proteolytic enzymes present in the polymorphonuclear neutrophil primary granules, released upon activation of the polymorphonuclear neutrophil and capable of degrading extracellular matrix proteins of the connective tissue (40). Higher granulocyte elastase activity has previously been observed in patients with periodontitis (both aggressive and chronic) in comparison to healthy controls (41), and our observations confirm these findings in that polymorphonuclear neutrophils from patients with aggressive and chronic periodontitis released significantly more elastase, even in the absence of any stimulation. Increased release of reactive oxygen species and

Table 2. Human neutrophil elastase (HNE) activity (mU) of polymorphonuclear neutrophils from subjects with aggressive and chronic periodontitis in comparison to periodontally healthy controls without stimulus and after contact with *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*

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 *P. gingivalis* 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 *A. actinomycetemcomitans* 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

*$p < 0.05$ in comparison with healthy controls at that time point.

elastase by polymorphonuclear neutrophils is likely to contribute to enhanced tissue destruction in periodontal disease (42). Ding *et al*. found species-specific differences in elastase release when polymorphonuclear neutrophils interacted with different periodontopathogens, and a large amount of elastase was measured after exposure to *Fusobacterium nucleatum*, but not when polymorphonuclear neutrophils were triggered with *P. gingivalis* or *Treponema denticola* (43).

The extracellular release of reactive oxygen species can cause lipid peroxidation (44) and may also result in damage to the surrounding tissues (45). Sheikhi *et al*. suggested that periodontal pathogens might stimulate polymorphonuclear neutrophils to release reactive oxygen species, and in their *in vitro* study, *F. nucleatum* was able to stimulate polymorphonuclear neutrophils to produce reactive oxygen species (44). The intracellular 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 phagocyctosis of *P. gingivalis,* superoxide was produced to a lesser extent compared with that seen after phagocytosis of *A. actinomycetemcomitans* (45). Our data confirm this observation in the context of patients with chronic periodontitis. Here, we measured less total and extracellular reactive oxygen species release (luminol-dependent and isoluminol-dependent chemiluminescence) when polymorphonuclear neutrophils phagocytosed *P. gingivalis* ATCC 33277 in comparison with *A. actinomycetemcomitans* Y4. Nevertheless, it should be mentioned that strain-dependent differences exist among *A. actinomycetemcomitans* strains in their ability to trigger the luminol amplified chemiluminescence (46).

Interestingly, the release of reactive oxygen species in the absence of any stimulation with *P. gingivalis* or *A. actinomycetemcomitans* was higher in polymorphonuclear neutrophils from patients with chronic periodontitis and aggressive periodontitis than in the periodontally healthy control group. This is supported by recent research of Matthews *et al*., who reported that polymorphonuclear neutrophils in chronic periodontitis were hyperactive in terms of extracellular release of reactive oxygen species without exogenous stimulation (47). In a further analysis they demonstrated that this release of reactive oxygen species in the absence of stimulation was also detectable after periodontal therapy (48). 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 (47,48). Furthermore, genotyping studies suggested that the disposition for hyperactive polymorphonuclear neutrophils may be a genetic risk factor for increased periodontal destruction (40).

While polymorphonuclear neutrophils from subjects with chronic periodontitis and from controls could be stimulated by *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4, the polymorphonuclear neutrophils from patients with aggressive periodontitis did not release additional reactive oxygen species after contact with those species. This observed lower reactive oxygen species release in aggressive periodontitis after contact with periodontal pathogens is in contrast to data reported by Shapira *et al*., who identified polymorphonuclear neutrophil hyperactivity after stimulation with opsonized bacteria in subjects with rapidly progressive periodontitis (now renamed as generalized aggressive periodontitis) (49). However, the difference between the findings of this study and our own may be a result of the different methods that were employed; we investigated the direct interaction between polymorphonuclear neutrophils and periodontal pathogens, whereas Shapira *et al*. (49) used streptococci opsonized with histone.

Conclusions

Within the limitations of our study, the results indicate that polymorphonuclear neutrophils 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 polymorphonuclear neutrophils. An extracellular release of reactive oxygen species and neutrophil elastase by polymorphonuclear neutrophils was detected and probably not only kills bacteria extracellularly but also damages the surrounding periodontal tissues.

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