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Relationship of neutrophil phagocytosis and oxidative burst with the subgingival microbiota of generalized aggressive periodontitis

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Introduction: Polymorphonuclear neutrophil (PMN) dysfunctions have been associated with severe forms of periodontitis. This study evaluated the correlation between PMN phagocytosis and oxidative burst with the subgingival microbiota of patients with generalized aggressive periodontitis (GAgP).

Methods: Heparinized peripheral blood samples were obtained from 18 GAgP patients and 11 periodontally healthy (PH) subjects, and PMNs were isolated on a Ficoll–Hypaque gradient. For phagocytosis analysis, PMNs were incubated with fluorescein-labeled *Staphylococcus aureus*. The oxidative burst was evaluated by incubation of PMNs with dihydroethidium and activation by *S. aureus*. The assays were examined using flow cytometry. Subgingival biofilm samples were obtained from periodontal sites with and without periodontitis and 24 species were detected by checkerboard.

Results: A significantly lower phagocytosis rate was observed for patients with GAgP compared with PH subjects over time (P < 0.05). No differences between groups were found for superoxide production. GAgP patients presented significantly higher prevalence and levels of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans* serotype b than controls (P < 0.05). Significant negative correlations between *T. forsythia* and *P. gingivalis* and PMN functions were observed. **Conclusions:** GAgP subjects presented diminished phagocytic activity of peripheral PMNs and high prevalence and levels of classical periodontal pathogens.

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Key words: aggressive periodontitis; checkerboard; flow cytometry; oxidative burst; phagocytosis; polymorphonuclear neutrophils; subgingival microbiota

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Aggressive periodontitis comprises a group of rare, often severe types of periodontal diseases that affect mainly young patients, occur in localized and generalized forms, have a rapid attachment loss and bone resorption, and are marked by familial aggregation (2). Over the years,

data supporting the microbial etiology of periodontal diseases have led to the recognition of several periodontally pathogenic species (18). Polymorphonuclear neutrophils (PMNs) form the first line of defense against these periodontal pathogens in the gingival sulcus (9). At this site, PMNs are actively involved in phagocytosing and killing bacteria through degranulation of enzymes and production of oxygen radicals. The phagocytosis of bacteria at this interface is enhanced dramatically by specific antibody recognition (47). There is clear evidence of the

importance of this protective PMN barrier, as patients with abnormalities in the numbers and/or functions of these cells often experience rapid and severe periodontal destruction (7). Several authors have reported a significant reduction in the chemotaxis, phagocytosis, and/or killing of bacteria by PMNs in patients with aggressive periodontitis (4, 11, 21, 28, 36, 45, 46). PMN dysfunctions have also been associated with recurrence of disease after treatment (25). The concept that PMNs are important protective cells is further supported by the fact that many periodontal organisms possess virulence factors that suppress neutrophil activities (11, 37). It remains unclear whether neutrophil impairment is an intrinsic characteristic or is a result of a predominant pathogenic subgingival microbiota in patients with severe destructive diseases. The current investigation aimed to measure and correlate peripheral PMN phagocytosis and anion superoxide production with the predominant subgingival microbiota of patients with generalized aggressive periodontitis.

Materials and methods Human subjects and clinical assessments

Twenty-nine adult subjects who sought dental treatment at the Dental School of the Federal University of Rio de Janeiro. Brazil, were recruited for the study. Informed consent was obtained from all enrolled individuals and the study protocol was approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital. Exclusion criteria included pregnancy, smoking, use of local or systemic antimicrobial agents within the 6 months before entry into the study, diabetes, and other systemic conditions that could affect the periodontal status. All subjects had at least 20 teeth and were over 18 years of age. Clinical measurements, including probing depth (PD), clinical attachment level (CAL), supragingival biofilm (SB) and bleeding on probing (BOP) were performed at six sites per tooth at all teeth, excluding the third molars, by one calibrated examiner. PD and CAL measurements were performed using a conventional manual periodontal probe (North Carolina Probe, Hu-Friedy, Chicago, IL). After initial clinical evaluation, the subjects were categorized into periodontally healthy (PH; n = 11; no sites with PD >3 mm and CAL >4 mm, and no more than 10% of sites with BOP); and generalized aggressive periodontitis patients (GAgP; n = 18;

more than three teeth other than first molars and incisors with PD and/or CAL ≥ 6 mm; $\geq 10\%$ sites with BOP).

Separation of PMNs

Peripheral blood was taken by venepuncture from each subject into a Vacutainer® tube containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Six milliliters of 4.2% dextran 70 (Sigma Chemical Co., St Louis, MO) was added to 15 ml heparinized blood. After incubation at 37°C for 45 min, PMNs were isolated by density gradient centrifugation at 200 gfor 45 min in 10 ml Ficoll-Hypaque (Sigma). The PMN pellet was collected and erythrocytes were removed by hypotonic lysis with 0.06 mM KCl in cold deionized water and Hank's medium without calcium and magnesium (Sigma). After centrifugation at 55 g for 10 min, PMNs were washed and resuspended in medium 199 (Sigma) containing 10% of 0.3 mM HEPES (Sigma). The number and viability of PMNs were determined in a Neubauer chamber (INLAB, Interlabor, São Paulo, SP, Brazil). Ninety microliters of Turk's solution were added to 10 µl of cell suspension. PMN viability was determined by the Trypan Blue exclusion test. More than 95% of PMNs were viable.

Fluorescein-labeled bacteria and opsonization

The strain Staphylococcus aureus ATCC 33591 was cultured in brain-heart infusion broth (BBL, Baltimore Cockeyville MD) at 37°C for 24 h. Bacteria were harvested by centrifugation at 10,000 g for 10 min, and killed by heating at 60°C for 1 h. After washing twice with phosphate-buffered saline (PBS), bacteria were resuspended in 0.1 M carbonate buffer (pH 9.6), and adjusted to 6×10^8 cells/ml. The bacterial suspension was labeled with 3 µg/ml of fluorescein isothiocyanate (FITC; Sigma) at 37°C for 30 min, washed three times in PBS, and fixed with 3.5% paraformaldehyde in PBS at 4°C for 1 h. Bacterial cells were then washed twice, resuspended in PBS, and the suspension was adjusted to 10⁸ cells/ml. Homogeneous, strong labeling was observed in 94.3% of S. aureus cells incubated with FITC (data not shown). For opsonization, serum samples were obtained from periodontally healthy subjects by centrifugation, then pooled and stored in sterile vials at -70°C. FITClabeled cells of S. aureus were opsonized by incubation with 50% pooled sera at 37°C for 30 min, washed twice, and resuspended in PBS to a concentration of 10^8 cells/ml (29).

Assessment of phagocytosis and oxidative burst

The kinetics of phagocytosis and oxidative burst of PMNs were analysed by flow cytometry with minor modifications of the method reported by Perticarari et al. (29). Briefly, PMNs at concentration of 10⁶ cells/ml were incubated with opsonized FITC-labeled bacteria at a ratio of 1:10 at 37°C for 60 min in a shaking water bath. A 700-µl aliquot was obtained every 15 min, washed once in cold PBS, and fixed with 1% paraformaldehyde in PBS. Extracellular FITC-labeled fluorescence was quenched by adding crystal violet (0.25 g/l in PBS; pH 7.3) to the samples. Data were acquired in a flow cytometer within 1 min after the addition of crystal violet. To assess the oxidative burst, PMNs (10⁶ cells/ml) were preincubated with 1 µl/ml of hydroethidine (Sigma) for 15 min at 37°C, and then incubated with opsonized but not labeled S. aureus at a ratio of 1 : 20 in a shaking water bath for 5 min. Aliquots were obtained at 0, 15, 40, and 60 min, fixed with 1% paraformaldehyde in PBS, and evaluated in the flow cytometer. All samples were acquired in a FACScaliburTM equipment (Beckton Dickinson, San Jose, CA), using the CellOuestTM software (Beckton Dickinson, Palo Alto, CA). Ten thousand cells were counted per sample by gating according to their characteristic forward and side scatters. Negative controls were carried out by inhibition of phagocytosis with cytochalasin B (Sigma) at a final concentration of 30 µg/ml in dimethylsulfoxide (Mallinckrodt Baker, Paris, KY), for 30 min at 37°C.

Microbiological assessment

Before the clinical examination, individual subgingival biofilm samples were taken from four sites with PD >5 mm and two sites with PD <4 mm and no BOP from each GAgP subject, and from two random sites with PD <4 mm and no BOP from each subject in the PH group. After removal of supragingival biofilm with sterile gauze, subgingival samples were individually obtained with sterile curettes. The presence and levels of 24 bacterial species (Table 1) were determined by the checkerboard DNA-DNA hybridization method (39). In brief, the samples were placed in separate Eppendorf tubes, the cells were lysed, and denatured DNA was

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Table 1. Subgingival species used for construction of whole genomic DNA probes tested against subgingival biofilm samples

Species	Strains ¹	Species	Strains ¹
Aggregatibacter actinomycetemcomitans a	43718	Propionibacterium acnes	11827
Aggregatibacter actinomycetemcomitans b	29523	Prevotella intermedia	25611
Actinomyces gerencseriae	23860	Porphyromonas gingivalis	33277
Actinomyces israelii	12102	Selenomonas noxia	43541
Actinomyces viscosus	43146	Streptococcus anginosus	33397
Tannerella forsythia	43037	Streptococcus constellatus	27823
Campylobacter rectus	33238	Streptococcus gordonii	10558
Capnocytophaga ochracea	33596	Streptococcus mitis	49456
Capnocytophaga sputigena	33612	Streptococcus oralis	33037
Eikenella corrodens	23834	Streptococcus sanguinis	10556
Fusobacterim periodonticum	33693	Treponema denticola	B1 ²
Fusobacterium nucleatum ss. vincentii	49256	Veillonella parvula	10790
Prevotella melaninogenica	25845	*	

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fixed in individual lanes on a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using the checkerboard slot blot device (Minislot 30; Immunetics, Cambridge, MA). Twenty-four digoxigenin-labeled (Roche Molecular Systems, Inc., Alameda, CA) whole genomic DNA probes were hybridized at 90° to the lanes of the samples using the hybridization device (Miniblotter 45; Immunetics). Bound probes were detected using phosphatase-conjugated antibody to digoxigenin (Roche Molecular Systems) and chemiluminescence (Amersham Pharmacia Biotech). Signals were detected on X-ray films (Kodak X-O MAT: Eastman Kodak Company, Rochester, NY), and evaluated visually by comparison with the standards at 10^5 and 10^6 bacterial cells for the test species on the same membrane. They were recorded as: 0, not detected; 1, $<10^5$ cells; 2, approximately 10^5 cells; 3, 10^5-10^6 cells; 4, approximately 10^6 cells; 5, $>10^6$ cells. The sensitivity of this assay was adjusted to permit detection of 10⁴ cells of a given species by adjusting the concentration of each DNA probe. This procedure was carried out to provide the same sensitivity of detection for each species. Failure to detect a signal was recorded as zero, although conceivably, counts in the 1 to 1000 range could have been present.

Statistical analysis

Statistical analyses were performed using the software Statistical Package for the Social Sciences (SPSS Inc.[®] v.13; Chicago, IL). Full-mouth clinical measurements were computed for each subject and then averaged across subjects within the groups. Using the software WINMDI v.2.8 (Joseph Trotter, Scripps, San Diego, CA), the fluorescence distribution was displayed as single histograms or dot plots. PMN phagocytosis as well as oxidative burst was expressed as intensity of fluorescence (amount of phagocytosis and cellular activation), and % of fluorescent phagocytes. Microbial data were expressed as mean % of colonized sites (prevalence) and mean counts $\times 10^4$ (levels) of each bacterial species, computed for the sampled sites in each subject, and across subjects within each group. Adjustments for multiple comparisons were made as described by Socransky et al. (38). Differences in demographic and clinical parameters between the two groups were sought using the Mann-Whitney and chi-squared tests. General linear model (GLM) analysis for repeated measures was used to compare the phagocytosis rate and anion superoxide production between groups over time. Associations between prevalence and levels of bacteria and PMN functions were tested using the bivariate Spearman (rho) coefficient correlation test, adjusted for multiple comparisons by the Bonferroni's test. Statistical significance was reached at a 5% level for all analyses.

Results

Clinical and demographic features

The demographic and clinical characteristics of the two groups are summarized in Table 2. No significant differences between the groups were observed regarding age (P = 0.308) and gender (P = 0.102); although there were more women in the GAgP group. Statistically significant differences between groups were observed for all periodontal parameters (Mann–Whitney test; P < 0.01), even when using gender as a covariate (GLM test; P < 0.01). Patients with GAgP presented severe periodontal destructive dis-

Table 2. Demographic features and full-mouth clinical parameters (mean \pm SEM) of the periodontally healthy subjects and patients with generalized aggressive periodontitis included in the study

		*
Clinical parameters	Periodontally healthy $(n = 11)$	Generalized aggressive periodontitis $(n = 18)$
Age in years (±SD)	27 ± 1.5	29 ± 1.2
% Men ²	44	16
Probing depth (PD) ^{1,3}	1.9 ± 0.04	4.1 ± 0.22
Clinical attachment level (CAL) ^{1,3}	1.9 ± 0.04	4.1 ± 0.19
% sites presenting:		
Supragingival biofilm ^{1,3}	33.8 ± 5	71 ± 4.4
Bleeding on probing ^{1,3}	11 ± 1.7	68 ± 5
$PD < 4 \text{ mm}^{1,3}$	100	51 ± 4.4
PD 4–6 mm ^{1,3}	0	35 ± 4
$PD > 6 mm^{1,3}$	0	14 ± 3
$CAL < 4 \text{ mm}^{1,3}$	98.6 ± 0.5	48 ± 4
CAL 4–6 mm ^{1,3}	1.4 ± 0.5	36 ± 4
CAL >6 mm ^{1,3}	0	16 ± 3.2

¹Refers to statistically significant differences between groups for the parameters evaluated (P < 0.01; Mann–Whitney test).

²Refers to the chi-squared test (no significant differences between groups were seen).

³Refers to statistically significant differences between groups for the parameters evaluated (P < 0.01; GLM test using gender as covariate).

ease as observed by the mean % of sites with PD (14 ± 3) and CAL $(16 \pm 3.2) > 6$ mm.

Phagocytosis and oxidative burst activities of PMNs

Figure 1(A,B) shows the phagocytosis of FITC-labeled bacteria expressed as mean (±SD) % of phagocytes and intensity of fluorescence, respectively, in the two subject groups after addition of crystal violet for quenching of fluorescent membranebound uningested bacteria. The number of phagocytosing PMNs was lower in patients with aggressive disease than in controls but the differences were not statistically significant (Fig. 1A; GLM, P = 0.524, using gender as a covariate). PMNs from individuals with GAgP showed a significantly lower rate of bacterial uptake compared with those from healthy control patients over time (Fig. 1B; GLM, P = 0.011, after controlling for gender). To confirm that differences in fluorescence intensity between

groups resulted from true phagocytosis, PMNs were treated with cvtochalasin B. which abolishes phagocytosis but does not affect bacterial adherence. An overall reduction of about 80% in the intensity of fluorescence was observed after treatment with cytochalasin B (data not shown). A 100% fluorescence reduction would not be expected because some bacteria may bind to the cell but are not internalized. The superoxide production is depicted in Fig. 2(A.B). Fig. 2(A) shows the mean %, and Fig. 2(B) shows the fluorescence intensity of PMNs producing reactive oxygen species (ROS) at different incubation periods. PMNs from both clinical groups demonstrated similar patterns of oxidative metabolism, and no significant differences were found (GLM, P = 0.484, after controlling for gender). As in the phagocytosis assay, the intensity of the red fluorescence resulting from the oxidative burst was validated by treating PMNs with cytochalasin B (data not shown).



Δ 100 % PMN presenting oxidative –∎–PH –∆–GAgP 80 60 burst 40 20 0 5 15 0 40 60 Time (min) В 600 HE florescence intensity 500 400 300 200 100 0 0 5 40 60 15 Time (min)

Fig. 1. Kinetics of phagocytosis rate of opsonized fluorescein isothiocyanate-labeled Staphylococcus aureus by polymorphonuclear neutrophils (PMNs) from patients with generalized aggressive periodontitis (GAgP) and periodontal healthy (PH) control subjects. (A) Percentage (mean ± SD) and (B) fluorescence intensity (mean \pm SD) of phagocytosing PMNs. All samples received crystal violet to assure that the fluorescence observed corresponded to ingested bacteria. In abscissa are indicated the incubation periods. Time 0 refers to the first reading after 60 min of incubation with labeled bacteria. *Refers to the repeated measure analysis of variance (general linear model), using gender as a covariate.

Fig. 2. Evaluation of the oxidative burst of polymorphonuclear neutrophils (PMNs) with hydroethidine (HE) from patients with generalaggressive periodontitis (GAgP) or ized periodontally health controls (PH) activated by non-labeled Staphylococcus aureus. (A) Percentage (mean \pm SD) and (B) fluorescence intensity (mean ± SD) of PMNs producing reactive oxygen species at different incubation periods. Time 0 refers to PMNs not activated with bacteria. No significant differences were found between groups over time; P > 0.05; repeated measure analysis of variance (general linear model), using gender as a covariate. For full species names see Table 1.

Microbiological findings

Most of the species tested were detected in higher prevalence in samples from patients with GAgP than in those from PH patients. except for Actinomyces viscosus, Actinomyces israelii, Campylobacter rectus, and Capnocytophaga ochracea (Fig. 3). The pathogens Aggregatibacter actinomycetemcomitans serotype b, Porphyromonas gingivalis and Tannerella forsythia were detected significantly more often in GAgP individuals compared with controls (Mann–Whitney test; P < 0.05, after adjustments for multiple comparisons). In contrast, A. actinomycetemcomitans serotype a, Fusobacterium spp. and Prevotella spp. showed a similar distribution in both groups. Species detected at low frequency included C. ochracea, Capnocytophage sputigena, Eikenella corrodens, Treponema denticola, Veillonella parvula and Streptococcus anginosus. Fig. 4 demonstrates the levels of the different species examined. Likewise, all species but Actinomyces gerencseriae and Prevotella melaninogenica were present at higher levels in GAgP patients than in controls. Significant differences were observed for A. actinomycetemcomitans serotype b, P. gingivalis, T. forsythia, A. viscosus, Propionibacterium acnes, Streptococcus constellatus, Streptococcus sanguinis and Streptococcus oralis (Mann–Whitney test; P < 0.05, after adjustments for multiple comparisons). In particular, the species C. sputigena and P. melaninogenica were observed at high prevalence in the GAgP group, but at elevated levels in control subjects. On the other hand, the PH group showed a high frequency of A. israelii, A. gerencseriae, and C. rectus, whereas GAgP patients harbored these species at increased counts (Figs 3 and 4).

Relationship between PMN activities and subgingival microbiota

Bivariate correlation analysis was performed and the Spearman rho coefficient was calculated to measure associations between each subgingival species and PMN phagocytosis and oxidative burst. The intensity of fluorescence at all timepoints was averaged for each subject in both assays. Of all the species tested, only the prevalence and levels of *T. forsythia* (rho = -0.612, P = 0.02 and rho = -0.561, P = 0.03, respectively), and *P. gingivalis* (rho = -0.500, P = 0.04 and rho = -0.638, P = 0.02, respectively) presented significant negative correlations



Fig. 3. Bar chart of mean frequency of detection (%) of subgingival species in generalized aggressive periodontitis (GAgP) and periodontally healthy (PH) subjects. *Refers to significance of difference in prevalence of bacteria between the two groups determined by the Mann–Whitney test, after adjusting for multiple comparisons (P < 0.05). #*A. actinomycetemcomitans* serotype b was not detected in any sample from PH individuals. For full species names see Table 1.

with the total rate of phagocytosis (Table 3). Regarding the oxidative metabolism, significant negative associations were found for levels of *A. viscosus* (rho = -0.693, P = 0.006), *T. forsythia* (rho = -0.634, P = 0.01), P gingivalis (rho = -0.702, P = 0.008), P acnes (rho = -0.644, P = 0.01), and Prevotella intermedia (rho = -0.567, P = 0.03). Although most of these correlation coefficients were relatively strong, when adjustments for multiple comparisons using the Bonferroni test were employed (adjusted P = 0.001), none of the associations were statistically significant.



Fig. 4. Bar chart of mean levels (× 10^4 bacterial cells) of subgingival species in generalized aggressive periodontitis (GAgP) and periodontally healthy (PH) subjects. *Refers to significance of differences in prevalence of bacteria between the two groups determined by the Mann–Whitney test, after adjusting for multiple comparisons (P < 0.05). [#]A. actinomycetemcomitans serotype b was not detected in any sample from PH individuals. For the small bars that cannot be visualized, labels with the corresponding values were added next to them.

Table 3. Correlation between polymorphonuclear neutrophil phagocytosis rate and oxidative burst and the mean prevalence (%) and levels ($\times 10^4$ cells) of subgingival bacterial species in the total subject population

Bacterial species	Rate of phagocytosis ³		Production of anion superoxide ³					
Mean prevalence (%)								
P.gingivalis	-0.500^{1}	0.04^{2}	-0.491^{1}	0.07^{2}				
T. forsythia	-0.612^{1}	0.02^{2}	-0.504^{1}	0.06^{2}				
Mean levels (×10 ⁴ cells	5)							
P.gingivalis	-0.638^{1}	0.02^{2}	-0.702^{1}	0.008^{2}				
T. forsythia	-0.561^{1}	0.03^{2}	-0.634^{1}	0.015^{2}				
P. intermedia	-0.082^{1}	0.78^{2}	-0.567^{1}	0.034^{2}				
P. acnes	-0.519^{1}	0.05^{2}	-0.644^{1}	0.013 ²				
A. viscosus	-0.212^{1}	0.47^{2}	-0.693^{1}	0.006^{2}				

¹Spearman rho coefficient.

²Not adjusted P values.

³Refers to average of fluorescence intensity over time for phagocytosis and superoxide production in each patient.

No significant correlations among species and PMN functions were seen after Bonferroni's adjustments for multiple comparisons (adjusted value at the 0.05 level P = 0.001).

Discussion

The present study evaluated the correlation between peripheral PMN phagocytosis and oxidative burst with the subgingival microbiota of patients with GAgP. Our data showed that GAgP patients presented a significantly diminished phagocytosis rate of opsonized bacteria compared with PH subjects. Similar findings have been reported by other investigators (4, 11, 21, 46). In contrast, some studies have not observed any significant difference in PMN phagocytosis between patients with periodontitis and controls (28, 41). Regarding the oxidative burst activity, available data suggest that peripheral PMNs from patients with different forms of periodontitis are 'hyperactive', and therefore release greater amounts of lysosomal enzymes and ROS than PMNs from individuals with periodontal health (12, 13, 16, 17, 19, 34, 35). This hyperactive phenotype may account in part for the tissue destruction seen in periodontitis (3). Nevertheless, it is still unclear whether this hyperreactivity is a constitutional dysfunction or a result of priming by various extrinsic factors such as a pathogenic periodontal microbiota and/or high levels of immunomodulatory cytokines (3). In the current investigation, a slightly lower production of anion superoxide was observed in PMNs from GAgP patients compared with controls by the method of ROS detection employed; however, no significant differences between groups were seen.

Conflicting data among our findings and other studies may have resulted from differences in study population, pathways of activation, and/or assays for phagocytosis analysis and ROS detection (3). One possible limitation of our study may have been the small size of the sample population. Lack of significance between groups in relation to gender, some measurements of PMN activity, and correlation with bacterial species could have been the result of the insufficient power of the study, so that careful interpretation of the data is necessary. Another aspect that should be considered in those studies is related to the criteria used for the diagnosis of periodontal disease or health. This has been a controversial issue in many clinical and microbiological studies in periodontology (44). There is a consensus that the classification and diagnosis of syndromic diseases such as periodontitis should be based on clinical, environmental, and laboratory parameters related to host-microbial interactions. However, at the moment the

definition of periodontal health or disease will continue to rely on well-described clinical criteria that characterize the level of disease of a particular population (44). We performed a full-mouth clinical examination to determine more precisely the periodontal status of each individual (44). In addition, we based our definition of health and disease on our database of over 500 patients examined in the last 5 years at the Federal University of Rio de Janeiro. as well as on epidemiological studies in Brazil (40). In our current database, for example, chronic periodontitis patients $(n = 253, \text{ mean age } 45.8 \pm 10 \text{ years})$ present mean PD and CAL of 3.1 ± 0.08 and 3.9 ± 0.1 mm, respectively, and $10 \pm$ 0.9% of sites with CAL >6 mm. In this study, the GAgP population comprised mainly young adult patients (mean age 29 ± 1.2 years) with severe periodontal destruction (mean PD and CAL 4.1 ± 0.2 ; $16 \pm 3.2\%$ of sites with CAL >6 mm) (Table 3), compatible with the diagnosis of GAgP (2).

Although no significant differences were found between groups regarding gender, female participants tended to be in greater proportions in the diseased group. The influence of gender on neutrophil functions remains uncertain; however, data using animal models indicated that sexual hormones seem to have different effects on the phagocytic activities of PMNs (15). In humans, it has been demonstrated that women with periodontitis present a more efficient neutrophil phagocytic activity than men (22). Comparisons of PMN functions between groups were controlled for the potential confounding effect of gender in the current study.

Regarding the methodology, we employed a flow cytometric method to measure phagocytosis and oxidative burst by PMNs (29). This technique allows the observation of events in a large number of cells per assay (21). Moreover, adherent bacteria can be distinguished from the ingested ones using a quenching agent like crystal violet. For detection of ROS, we employed the compound hydroethidine. Oxidation of hydroethidine.measures mainly the production of superoxide anion (O_2^{-}) , the primary metabolite generated during respiratory burst (31). Fredriksson et al. (12) showed a higher oxidative burst in periodontitis patients compared with controls when the total generation of oxygen radicals was measured, but no differences were found when only intracellular H2O2 was assessed. Assessment of respiratory burst should take into consideration the production of all ROS rather than relying only on one specific metabolite.

Studies on PMN phagocytosis and oxidative burst have also indicated that the increased ROS production by neutrophils from periodontitis patients is related to complement or Fc receptor stimulation with immunoglobulin G opsonized bacteria (12, 13, 16, 17, 19). In addition, differences in Fc receptor genotypes have been shown to affect PMN functions. Kobayashi et al. (23) showed that PMNs from subjects with chronic periodontitis or periodontal health carrying the FcyRIIIb-NA2 allele present a less efficient phagocytosis and oxidative burst induced by opsonized bacteria. Other investigators (26) demonstrated that individuals with periodontitis who have the H/H genotype have a hyperreactive PMN phenotype, with increased Fcy-mediated phagocytosis, degranulation and granular enzyme release. We have recently evaluated the distribution of FcyR genotypes in GAgP patients and healthy controls from the same population. No significant differences between groups were found regarding the H/R genotypes. However, the FcyRIIIb-NA2/NA2 genotype was significantly overrepresented in GAgP patients (8). This could explain, in part, the lower phagocytosis rate and superoxide production observed in patients with aggressive periodontitis compared with controls in this population.

Microbiological data demonstrated that GAgP patients harbored significantly higher prevalence and higher levels of putative periodontal pathogens than healthy controls, particularly the species P. gingivalis, T. forsythia and A. actinomycetemcomitans serotype b. A. actinomycetemcomitans has been associated with aggressive forms of periodontitis (30, 43, 50); however, other investigations have reported low prevalence and proportions of this species in GAgP patients (20, 42, 49). The mean frequency and levels of P. gingivalis and T. forsythia were strikingly elevated in our patients with aggressive periodontitis. These findings are in accord with previous studies that indicated the relevance of these pathogenic species in GAgP (24, 42, 49).

The association of these organisms with aggressive disease is further supported by the negative correlations observed between their frequency and levels and PMN functions (Table 3). Although these correlations do not imply causation, it can be hypothesized that the *in vitro* decreased phagocytosis rate and

ROS production of peripheral PMNs may favor the overgrowth of a more pathogenic subgingival microbiota in GAgP subjects. Conversely, our data do not provide evidence of a suppressive effect of this microbiota on peripheral neutrophil functions given the fact that we used a non-oral bacterial species (S. aureus) instead of putative periodontal pathogens for measuring PMN activities. Nevertheless, it is a fact that several periodontal pathogens elaborate various antineutrophil virulence factors which increase their resistance to phagocytosis (32, 33, 37, 48), and to oxidative killing by PMNs (1, 6, 10, 14). In addition, at the periodontal site phagocytic cells may interact with bacterial species arranged in a biofilm structure. Species living within biofilms are notoriously resistant to killing by host defense mechanisms and antibiotics (5). For instance, Ochiai et al. (27) demonstrated that co-aggregated cells of Streptococcus mitis and A. viscosus were more resistant to phagocytosis and killing by neutrophils in vivo and in vitro than pure suspensions of these organisms.

It should be pointed out that our main goal in this study was not to evaluate the direct effects of the subgingival microbiota on PMN functions, but rather to determine whether an intrinsic impairment in these cells would be associated with a pathogenic periodontal microbiota and disease. To date, very few studies have correlated the subgingival microbiota with PMN activities in individuals with aggressive disease (28, 36). Moreover, there is not a unique test to measure phagocytic or microbicidal functions of PMNs in the gingival sulcus because a wide variety of subgingival pathogens interact simultaneously with a large number of different components on the surface of these cells, eliciting distinct responses. In vitro interactions between PMNs and specific periodontal pathogens do not necessarily mimic the in vivo mechanisms that take place in the oral microenvironment.

In conclusion, this investigation indicates that peripheral neutrophils from individuals with GAgP present a depressed phagocytosis rate of opsonized bacteria. Furthermore, significantly higher prevalence and levels of periodontal pathogens are observed in the subgingival microbiota of these patients in relation to controls. Conceivably, impairment of PMN phagocytosis in GAgP patients may favor the establishment of a more pathogenic microbiota in these individuals.

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