Decreased phagocytic function in neutrophils and monocytes from peripheral blood in periodontal disease

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ABSTRACT

Phagocytosis by neutrophils and monocytes constitutes the main defense mechanism against bacterial challenges in periodontitis. Phagocytosis by neutrophils has already been evaluated, whereas phagocytic function of monocytes has hardly been addressed so far. Objectives: The aim of this study was to assess phagocytosis by neutrophils and monocytes in periodontitis. Material and Methods: The sample included 30 subjects with severe periodontitis and 27 control subjects without periodontal disease. The phagocytic index (PhI) was calculated as the mean number of adhered/ingested *Saccharomyces cerevisiae per* phagocytozing monocyte or neutrophil multiplied by the percentage of phagocytes involved in phagocytosis. Results: A significant reduction in phagocyte functions was observed in individuals with periodontitis. The median of PhI of neutrophils using non-sensitized *S. cerevisiae* was 3 for the control group, and 1.5 for the periodontitis group (p=0.01, Mann-Whitney test). The median of PhI of monocytes with non-sensitized *S. cerevisiae* was 26.13 for the control group, and 13.23 for the periodontitis group (p=0.03, Mann Whitney test). The median of PhI of monocytes assessed with sensitized *S. cerevisiae* was 97.92 for the control group and 60.1 for the periodontitis group (p=0.005, t-test). Conclusion: The data demonstrated a reduction in the function of phagocytes, suggesting a decrease in immune defenses in periodontitis.

Key words: Periodontitis. Neutrophils. Monocytes. Phagocytosis.

INTRODUCTION

It has been considered that periodontal infection or periodontitis results from the imbalance between the direct and indirect effects of pathogenic bacteria and the host immune response[7]. Data support the microbial etiology of periodontal disease and the role played by specific pathogenic species[5,25]. Polymorphonuclear (PMN) neutrophils constitute the first line of defense against these pathogens in the subgingival area. In the presence of plaque, an inflammatory infiltrate is frequently in the gingival tissues, showing mononuclear phagocytes, lymphocytes, and polymorphonuclear leukocytes[16,22]. Additionally, inflammatory response changes the microenvironment of the biofilm and selects for specific organisms[28]. The interaction between lipopolysaccharide (LPS) and these predominant cell types in the inflammatory infiltrate stimulates the production and elevation of prostanoids, mainly prostaglandin E_2 in the gingival fluid of sites that present attachment loss[30]. In addition to the local effects, the bacterial virulence factors and inflammatory mediators arising from this parasite-host interaction may create and sustain a chronic systemic inflammatory process in the bloodstream[17]. In the most aggressive forms of periodontal disease, the role played by the elevated serum levels of tumor necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1) has been considered partly

responsible for altering the neutrophil function[1]. In addition, new perspectives have indicated that periodontitis occurs as a hyperactive immune/inflammatory response to the specific bacteria of plaque in predisposed individuals, involving the excessive generation of oxygen radicals and the release of proteases[14]. However, despite this new paradigm that PMN is not "hypofunctional" or "deficient", but "hyperfunctional"[14], phagocytosis in this context is not yet elucidated.

Data on phagocytosis by neutrophils from peripheral blood in individuals with periodontitis are controversial. There are reports both of reduction[2,4,12,29] and increase of phagocytosis by neutrophils[13,21]. Studies addressing the phagocytosis by monocytes in these patients are still scarce. Therefore, it still has to be determined whether phagocytosis by monocytes in individuals with periodontitis is different compared to individuals without periodontal disease. For this reason, it is important to investigate the immune function represented by phagocytosis in periodontitis to gain insight into the defense against pathogens involved in disease conditions and the pathophysiological mechanism involved, including those of infectious nature, which are present during the course of the disease. Therefore, this study evaluated the phagocytic function of monocytes and neutrophils in periodontal disease, in comparison with control individuals without periodontal disease.

MATERIAL AND METHODS

Subjects and study groups

This study was approved by the Institutional Review Board of the Health Sciences Faculty – UnB – University of Brasilia (045/2008). The subjects were evaluated and selected for inclusion in the present study at the periodontal clinic of the University Hospital of Brasilia, Brazil. The sample included 30 subjects with periodontal disease and 27 subjects with healthy periodontium, all otherwise healthy and non-smokers. The periodontitis group consisted of 20 women and 10 men (age range 21-45 years, mean age 34.5 years) with at least 18 present teeth. The severe periodontitis was diagnosed according to the following inclusion criteria[23]: radiographic evidence of bone loss extending to $\geq$30% of the root length in multiple teeth, age $\geq$18 years, presence of $\geq$2 teeth/quadrant with a pocket depth of $\geq$6 mm and concomitant attachment loss of >3 mm. The control group consisted of 18 women and 9 men (age range 21-44 years, mean age 34 years) with clinical probing depths (PD) ≤3 mm and clinical attachment level (CAL) ≤3 mm, ≤10% sites with bleeding on probing and no radiographic evidence of bone loss. The following exclusion criteria were considered: previous mechanical periodontal therapy and antimicrobial therapy for systemic or topical oral use in the last 12 months, pregnant or lactating women, diabetes, morbid obesity, autoimmune, infectious, allergic, and gastrointestinal diseases, malnutrition, renal alterations, cancer or any other clinical situation that might alter the function of the immune system, use of medications that could alter the level of inflammatory mediators, and smoking.

Clinical examination

The initial periodontal evaluation of each patient included periapical radiographic documentation by the parallelism technique. The clinical examinations were performed by an experienced examiner and included visible plaque accumulation (PI) without the use of any disclosing agent, bleeding on probing (BOP), probing depth (PD) and clinical attachment level (CAL). The measurements were assessed at four sites around each tooth, namely buccal, lingual and proximal sites (the greatest depth was recorded for each proximal surface) using a manual probe (Michigan O probe with Williams markings), excluding third molars. Analyses of degrees of mobility and furcation involvement were recorded.

Phagocytosis test

Phagocytosis of *Saccharomyces cerevisiae* was adapted from a technique previously described[19]. Briefly, samples of 40 µL *per* marked area of heparinized whole peripheral blood obtained by venipuncture from each subject were placed on clean glass slides containing 8 marked areas with 7-mm diameter each, in duplicate preparations, and incubated in a wet chamber for 45 min at 37°C. The slides were then rinsed with 0.15 M phosphate-buffered saline (PBS) pH 7.2 at 37°C to remove non-adherent cells. After washing, neutrophils and monocytes remained adhered onto the slide approximately in the same proportion as they were in the whole blood. Adherent cells (12,534±5,050 cells/marked area; 5.63±0.85% monocytes and 93.5±1.08% neutrophils) were incubated with a suspension of $2.5x10^5$ *S. cerevisiae* in 20 µL Hanks-tris (Sigma Co., St Louis, MO, USA) pH 7.2, with 10% heat-inactivated fetal calf serum (FCS) (Gibco/Invitrogen, Grand Island, NY, USA) for 30 min in a wet chamber at 37°C. To evaluate the influence of complement molecules on phagocytosis in periodontitis the *S. cerevisiae* were incubated at 37°C for 30 min with 10% fresh serum from the donor in Hanks-Tris solution. Slides were then rinsed with 0.15 M PBS at 37°C to eliminate nonphagocytosed *S. cerevisiae* and the final washing was done with 30% FCS in Hanks-tris. The slides were fixed with absolute methanol and stained with 10% Giemsa solution. The number of *S. cerevisiae* phagocytozed by 200 monocytes or by 200 neutrophils in individual preparations was

assessed by light microscopy. Microscopic fields distributed throughout the slide were randomly selected and all monocytes or neutrophils in each particular field were examined. The PhI was calculated as the mean number of phagocytozed *S. cerevisiae per* phagocytosing monocytes or neutrophils, multiplied by the percentage of these cells engaged in phagocytosis[19].

Baking yeast (*S. cerevisiae*) was prepared according to a technique previously described[20].

Statistical analysis

Statistical analysis was performed using the Prism® software, 2005 (Graphpad, San Diego, CA, USA). Beforehand the variables in the samples were previously verified for normality, using the Skewness and Kurtosis and Kolmogorov-Smirnov tests. The t-test was used for comparison between two variables with normal distribution, and the Mann-Whitney test was used for those that did not present normal distribution. The differences between variables were considered statistically significant when the bi-caudal probability of their occurrence due to chance (error type I) was lower than 5% ($p<0.05$). As several data showed non-normal distribution, for homogeneity, all data were graphically expressed as median, quartiles and extremes.

RESULTS

Clinical and demographic characteristics

The clinical and demographic characteristics of the two groups are summarized in Table 1. Distribution of age and gender was similar between control and periodontitis groups. Subjects with periodontitis had significantly higher body mass index than control ($p=0.001$). Statistically significant differences were also observed for all periodontal parameters. Subjects with periodontitis showed severe destructive periodontal disease when observed by the percentage of sites with PD $\leq$4 mm to $\geq$7 mm and CAL $\leq$4 mm to $\geq$7 mm. The hematological characteristics of the groups are listed in Table 2. No statistically significant difference was observed between groups, except for C reactive protein (CRP). The mean of serum levels of CRP was 0.21±0.25 for the control group and 0.51±0.62 for the periodontitis group ($p=0.01$).

Phagocytosis test

PhI of neutrophils and monocytes for non-opsonized *S. cerevisiae*

The median of the PhI of neutrophils from individuals with periodontitis (1.5) was significantly lower than that of normal controls (3.0), ($p=0.01$, Mann-Whitney test) (Figure 1A). This occurred

Table 1- Demographic characteristics and full-mouth clinical parameters

Characteristics/ Parameters	Control (n=27)	Periodontitis (n=30)	(p) Test
Age (years; mean+SD)	33.2±6.4	33.5±6.8	0.8441**
Gender (males/females: n)	9/18	10/20	1.0000***
Numbers of teeth (mean+SD)	28±1.3	27.5±4.8	0.3545*
BMI	20.3±1.28	26.53±5.49	0.0001*
SBP(mmHg)	120.9 ± 4.8	122.4±14.8	0.9188*
DBP (mmHg)	80.5±2.5	80.9±11.6	0.0744*
PI (%; mean+SD)	4.8±2.1	61.37±33.59	0.0001*
BOP (%; mean+SD)	2.7±1.2	41.83±30.04	0.0001*
PD (mm; mean+SD)			
≤4 mm	100%	3.8±3.97	
5-6 mm	0%	16.1±9.33	
≥7 mm	0%	9.8±8.8	
CAL (mm; mean+SD)			
≤4 mm	100%	5.8±4.5	
5-6 mm	0%	17.9±9.2	
≥7 mm	0%	13.0±11.4	

* Mann-Whitney test

** t-test

*** Chi-Square test, BMI (body mass index), SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure) PI (plaque index), BOP (bleeding on probing), PD (probing depth), CAL (clinical attachment level).

Table 2- Biochemical and hematological characteristics

Characteristics/ Parameters	Control (n=27)	Periodontitis (n=30)	(p) Test
Triglycerides (mg/dL)	86.1±35.4	98.9±46.2	0.2675*
Total cholesterol (mg/dL)	172±31.4	173.9±3.3	0.8222**
HDL-cholesterol (mg/dL)	48.2±12	42.5±12.3	0.0747*
LDL-cholesterol (mg/dL)	106±24.4	111.5±27.3	0.4348**
Glucose (mg/dL)	85.3±6.7	90.6±14.1	0.1528*
Eosinophils	146.1±103.3	222.9±175.8	0.0646*
Basophils	14.7±31.8	8.1±21.3	0.627*
Lymphocytes	2,239±530.7	2,137±499.5	0.4607**
Monocytes	422.6±140.9	388.5±160.1	0.2369*
Total leukocytes	6,093±1,216	6,308±1.473	0.5111*
C-Reactive Protein	0.21±0.25	0.51±0.62	0.0105*

* Mann-Whitney test

** t-test

because there was a reduction in the percentage of neutrophils involved in phagocytosis in the periodontitis group (0.50%) when compared to the normal control group (2.25%) (p=0.006, Mann-Whitney test) (Figure 1C), whereas no statistically significant difference was observed between groups for the mean number of yeasts adhered to/ingested by neutrophils (p=0.50, Mann-Whitney test) (Figure 1B).

The same situation was verified when phagocytosis by monocytes was tested. The median of the PhI of monocytes from individuals with periodontitis (13.23) was significantly lower than that of the normal controls (26.13) (p=0.02, Mann-Whitney test) (Figure 1D), because a statistically significant reduction in the percentage of monocytes involved in phagocytosis was observed in the periodontitis group (8.50%) when compared to the control group (17.25%) (p=0.01, Mann Whitney test) (Figure 1F). Conversely, no difference was observed between groups for the mean number of yeasts adhered to/ingested by monocytes (p=0.71, t test) (Figure 1E).

PhI of neutrophils and monocytes for opsonized *S. cerevisiae*

A different situation occurred when phagocytosis was evaluated using sensitized *S. cerevisae*. No statistical difference was observed in the PhI of neutrophils between individuals with periodontitis (117.9) and normal control individuals (157.0) (p=0.14, t-test) (Figure 2A). Periodontitis had no influence on the mean number of yeasts adhered/ingested by neutrophils (p=0.15, Mann-Whitney test) as illustrated in Figure 2B. There was no statistical difference in the involvement of neutrophils in phagocytosis between the two study groups (p=0.16, Mann-Whitney test) (Figure 2C).

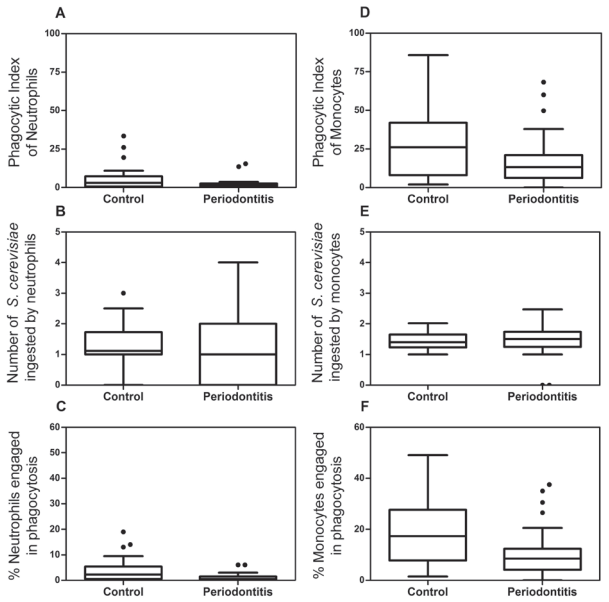

Figure 1- *In vitro* evaluation of the phagocytic capacity of neutrophils (left) or monocytes (right) in individuals with periodontal disease (P) and normal control individuals, using $2.5x10^5$ non sensitized yeast *per* well. A: Reduction of the phagocytic index (p=0.01, Mann-Whitney test). B: Mean number of yeasts adhered to/ingested by neutrophils (p=0.50, Mann-Whitney test). C: Reduction of the percent of neutrophils involved in phagocytosis (p=0.006, Mann-Whitney test). D: Reduction of the phagocytic index of monocytes (p=0.0,2, Mann-Whitney test). E: Mean number of yeasts adhered to/ingested by monocytes (p=0.71, t-test). F: Reduction in the percentage of monocytes involved in phagocytosis (p=0.01, Mann-Whitney test). Data were expressed as median, quartile and extreme. Outlier values are marked

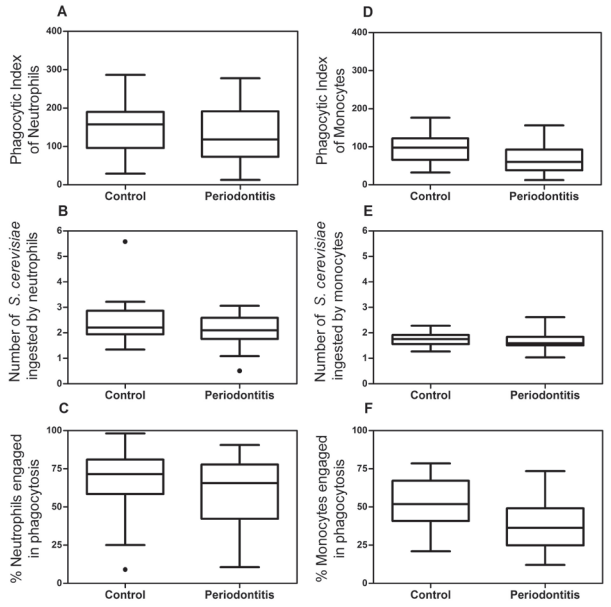

Figure 2- *In vitro* evaluation of the phagocytic capacity of neutrophils (left) or monocytes (right) in individuals with periodontal disease (P) and normal control individuals, using 2.5x10^5 sensitized yeast *per* well. A: Phagocytic Index (p=0.14, t- test). B: Mean number of yeasts adhered to/ingested by neutrophils (p=0.15 Mann-Whitney test). C: Percentage of neutrophils involved in phagocytosis (p=0.16, Mann-Whitney test). D: Reduction of the Phagocytic Index of monocytes (p=0.005, t- test). E: Mean number of yeasts adhered to/ingested by monocytes (p=0.19, Mann-Whitney test). F: Percentage of monocytes involved in phagocytosis (p=0.001, t-test). Data were expressed as median, quartile and extreme. Outlier values are marked

The median proportion of neutrophils involved in phagocytosis in the periodontitis group was 65.50%, compared to 71.50% in the control group.

However, a different result was verified when phagocytosis was tested by monocytes, where the median of PhI of monocytes from individuals with periodontitis (60.10) was significantly lower than that from the normal controls (97.92) (p=0.005, t-test) (Figure 2D). This decrease was caused by a lower percentage of monocytes involved in phagocytosis, namely 36.25% for the periodontitis group and 51.75% for the control group (p= 0.0016, t-test) (Figure 2F). The medians of the mean number of yeasts adhered to/ingested by monocytes were statistically similar between both groups, as illustrated in Figure 2E.

DISCUSSION

This is the first description of decreased phagocytic capacity of monocytes in human periodontal diseases. In the present study phagocytosis by monocytes and neutrophils were compared between individuals with healthy periodontium and those with periodontitis. In this work, killed *Saccharomyces cerevisiae* was used because receptors involved in their uptake are involved in phagocytosis by neutrophils and monocytes of pathogenic bacteria present in periodontal disease[27]. When using live bacteria, their virulence factors may influence phagocytosis. Thus, when investigating the phagocytosis, two lines of reasoning can be defined: the direct action of bacteria in phagocytosis and the effects of host-parasite interactions in phagocytosis. As our aim was to evaluate the effects of this interaction in the host, this justifies another stimulus provided to the cell, including yeasts. The use of different stimuli is reported in the literature: N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)[29], *Staphylococcus aureus*[5], *Candida albicans*[2,25], *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*[13,21].

The present study showed a significant reduction in the phagocytic capacity of monocytes, both using opsonized and non-opsonized *S. cerevisiae* (Figure 1D and 2D). However, for neutrophils, the disease decreased phagocytosis only when it was assessed using non-opsonized yeasts (Figure1A). This lower phagocytic capacity of neutrophils in periodontitis has also been reported by other researchers[2,4,12,29]. In this study, lower phagocytosis using non-opsonized *S. cerevisiae* was observed in the group of individuals with periodontitis, both by neutrophils (Figure 1A) and monocytes (Figure 1D). These data suggest that the phagocytic function of both neutrophils and monocytes was intrinsically affected because, in the absence of serum components, the level of phagocytosis was lower in the periodontitis group. Regarding the monocytes, for both analyses, the PhI was lower in individuals with periodontal disease (Figure 1D and 2D). A reduced number of monocytes involved in phagocytosis in this group was also evident (Figure 1F and 2F).

The mechanisms for the deficient response of phagocytes have not been explained yet, however some hypotheses can be suggested. It is known that many periodontal pathogens develop particular strategies for subverting the mechanisms of phagocytosis[27]. Among the mechanisms of immunosuppression, the role of LPS has been considered. Studies have indicated that the LPS of *P. gingivalis* appears to be antagonists of the toll-like receptor-4, thus competing with LPS of other species to couple with that receptor. This is a possible mechanism of deficiency of the innate immune system of the host, since recognition of the bacterial pathogens identified by the toll-like receptor-4 would be blocked[6]. The recruitment of neutrophils and macrophages in mice infected with *Aa* and lacking the toll-like receptor-2 led to the

reduction in the influx of these cells in the peritoneal cavity. Infection with *Aa* in this experimental model caused a significant decrease in the cytokine and chemokine levels and reduction in the phagocytic capacity of neutrophils and monocytes, in addition to alveolar bone loss[11].

Another possibility that has been described is the direct toxicity of *Aa* to neutrophils and monocytes by the production of leukotoxin[26]. Strains of *Aa* have various mechanisms that control phagocytosis, such as inhibition of chemotaxis and immunosuppressive and cytotoxic factors that suppress both the unspecific and specific immune responses, as well as preventing fibroblast proliferation. They also present the additional capacity to invade epithelial and endothelial cells[15]. Baehni, et al.[3] (1979) observed by electron microscopy that the cytotoxic effects on PMN by strains of *Aa* were independent of phagocytosis. The authors suggested that soluble bacterial products may be released by bacteria. Carvalho, et al.[4] (2009) found high frequency of *P. gingivalis, Tannerella forsythia* and *Aa* in individuals with aggressive periodontitis. They observed that in these individuals the frequency and quantity of *P. gingivalis* and *T. forsythia* presented negative correlation with phagocytosis by PMN. The depression of phagocytosis observed in the present study in the periodontal disease group can be justified by the action of these pathogens usually found in more severe forms of periodontal disease. In the analysis of phagocytosis by neutrophils using yeasts sensitized with fresh serum from the individual, although the PhI in individuals from the periodontitis group presented a trend toward reduction, the results did not differ statistically from those of the control group (Figure 2A). This suggests that there was no considerable change in complement an immunoglobulin receptors on neutrophils, as well as considerable influence of other serological factors in this group.

Different from the present results, studies in individuals with more severe forms of periodontal disease demonstrate that regulatory factors in serum may modulate functions of PMNs. Depression of the chemotactic response in patients with localized aggressive periodontitis may not be an abnormality associated with the cell, but rather a consequence of the elevation of the serum concentration of cytokines produced during the host-parasite interaction. The TNF-alpha and IL-1 cytokines may cause a reduction in chemotactic receptors[1]. However, increased local production of cytokines in response to pathogens in the periodontium may result in bone loss and tissue damage typically observed in more severe forms of periodontitis[10,30]. Although there are no studies showing the relationship between serum levels of inflammatory mediators and phagocytosis, there may be an inverse correlation between them because depressed phagocytosis is always found in periodontal pockets[9,24].

Although with small difference, the present data demonstrated that the serum level of C-reactive protein (CRP) was increased in individuals with periodontitis (Table 2). In more severe forms of periodontal disease, the association between the LPS of periodontopathogenic bacteria and inflammatory mediators has been well established[8], which leads to the increase of CRP with consequent cardiovascular alterations[18]. Similarly, it is possible that systemic immunosuppression of phagocytic cells, as observed in this study, may reduce the defense against bacteria and fungi. It is also a possibility the fact that the absence of systemic manifestations, observed in the present study, occur due to the redundancy of immune system functions. The long-term consequences of the reduction of phagocytosis in the group with periodontitis should be further evaluated.

CONCLUSIONS

This study showed for the first time that the monocytes of peripheral blood from individuals with periodontitis present decreased phagocytosis of opsonized and non-opsonized *S. cerevisiae* in comparison with control individuals. Concerning the neutrophils, decreased phagocytosis was observed only for non-opsonized yeasts. Although the individuals did not show clinical parameters of immunodeficiency, a laboratorial decreased function of phagocytes was characterized in this work, which may be a consequence, not the cause, of periodontitis. More studies should be conducted to investigate the immune inflammatory events implicated in phagocytosis by neutrophils and monocytes in periodontitis.

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