A Hyperactive Neutrophil Phenotype in Patients With Refractory Periodontitis

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Background: Neutrophils (PMNs) are critical components of the innate immune system and help to maintain oral health in the face of a constant bacterial challenge. However, along with protecting the periodontium from microbial invasion, these cells release potent lysosomal enzymes and oxygen radicals that can be destructive to periodontal tissues and lead to tooth loss. We examined neutrophil function in a unique population of patients diagnosed with refractory aggressive periodontitis (RAP).

Methods: Venous blood was obtained from 12 non-smoking patients who had been diagnosed with RAP, 10 patients with chronic periodontitis who had responded to periodontal therapy (CP), and 13 periodontally healthy controls (HCs). Peripheral blood PMNs were loaded with dihydrorhodamine 123 and stimulated with phorbol 12-myristate 13-acetate (PMA) to measure the receptor-independent respiratory burst of these key immune cells. Phagocytosis via the complement and Fc-gamma receptors was also assessed.

Results: PMNs from patients with RAP displayed significantly increased PMA-induced oxygen radical production compared to those from the HC and CP patients. PMNs from RAP patients also displayed increased phagocytosis compared to those from the CP group.

Conclusions: Our findings demonstrated a larger receptor-independent respiratory burst and higher phagocytotic activity in PMNs derived from patients with RAP compared to PMNs derived from CP patients and periodontally HCs. We speculate that the higher intrinsic intracellular activity of the nicotinamide adenine dinucleotide phosphate oxidase system may account for the continued periodontal breakdown, despite ongoing periodontal therapy in these challenging patients. *J Periodontol 2007;78:1788-1794.*

KEY WORDS

NADPH oxidase; neutrophils; phorbol myristate acetate; refractory periodontal disease; respiratory burst.

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Periodontitis is an inflammatory disease that results in destruction and degradation of the soft and mineralized connective tissues that support and house the dentition. It is widely accepted that the initiation and progression of periodontal disease are caused by the presence of pathogenic microorganisms that invade the host.[1] These microorganisms can cause direct damage to the periodontium; they also cause damage indirectly by activating a variety of host-mediated pathways that result in connective tissue destruction.

Neutrophils (polymorphonuclear leukocytes [PMNs]) are critical components of the innate immune system and help to maintain oral health in the face of a constant oral bacterial challenge.[2] PMNs are the first line of defense against a microbial challenge to the periodontium[3] and serve a protective function through their ability to phagocytose and kill microorganisms.[4] However, along with protecting the periodontium from microbial invasion, PMNs release potent lysosomal enzymes and oxygen radicals that can be destructive to the periodontal tissues.[5]

The vital role that PMNs play in protecting and maintaining the periodontium is evident in the oral manifestations present in patients with known qualitative and/or quantitative neutrophil disorders, such as benign neutropenia, leukocyte adhesion deficiency type I, and agranulocytosis. Although these diseases may have different clinical presentations and etiologies, they are all associated with

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varying degrees of susceptibility to infection that manifest orally as severe aggressive periodontitis and the premature loss of primary and permanent teeth.[1]

It was suggested that one cause of the destructive process evident in chronic periodontitis is the "hyperactivity" of neutrophils, resulting in overproduction of antimicrobial and potentially tissue-damaging oxygen free radicals.[6] However, it has yet to be determined if the neutrophils from patients with periodontitis are intrinsically different from those of healthy patients or if their hyperactivity is a result secondary to the periodontal inflammation itself.

The resting neutrophil depends upon anaerobic glycolysis for energy; however, once stimulated the oxygen consumption of the cell increases 100-fold.[7] This is known as the respiratory or oxidative burst of the neutrophil. The enzyme complex responsible for the oxidative burst is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase enzyme complex is composed of at least five members, two of which are stored within the cytosol and three that are membrane bound.[8] The members of the NADPH oxidase enzyme are dissociated and inactive in resting neutrophils and assemble together upon activation to form the active oxidase. NADPH oxidase removes an electron from molecular oxygen, resulting in the production of superoxide anions (O_2^-). The resulting superoxide anion (O_2^-) itself is potentially toxic; however, most of it is converted into hydrogen peroxide by a cytoplasmic enzyme known as superoxide dismutase. The neutrophil can release the enzyme myeloperoxidase, which is stored in the primary (azurophilic) granules, to convert hydrogen peroxide into extremely toxic hypoclorous acid. Hypoclorous acid is considered to be the primary oxygen radical responsible for neutrophil-mediated cytotoxicity.[9] Oxygen radicals produced by the neutrophils are considered to be antibacterial in nature; however, they also have the ability to attack host cell and tissue components and can be responsible for modulating various cellular activities that initiate tissue injury.[10]

Most forms of periodontal disease are treated predictably by conventional periodontal therapy.[11] Once treated, most patients can preserve their dentition with proper maintenance for long periods of time.[12,13] However, a number of patients with periodontal disease fail to respond to treatment and continue to show periodontal breakdown, despite what has been recognized as proper and adequate periodontal therapy,[14] leading researchers and clinicians to believe that there is a systemic component unique to these patients that may be a factor in the pathogenesis of this condition.[15] These patients may be considered "refractory" to treatment.

Patients characterized as unresponsive to multiple periodontal therapies pose a significant challenge to practitioners.[16] The ability to identify patients who may be unresponsive to conventional periodontal therapy because of intrinsic PMN defects will help in treatment planning and will allow us to understand the underlying mechanisms associated with continued periodontal destruction in this group of challenging patients. It is our hypothesis that patients with refractory aggressive periodontitis (RAP) have altered neutrophil functions compared to patients with successfully treated chronic periodontitis (CP) and periodontally healthy controls (HCs) and that this alteration may prove to be one cause of the apparent lack of periodontal stability and continual periodontal breakdown, despite ongoing therapy in these patients.

The aim of this study was to compare the generation of oxygen radicals in peripheral PMNs from patients with RAP, CP, and periodontally HCs after stimulation with phorbol myristate acetate (PMA). The second aim of the study was to examine the phagocytotic ability of the neutrophils.

MATERIALS AND METHODS

Study Population

All study subjects were patients who presented regularly to the Faculty of Dentistry, University of Toronto, for treatment and/or consultation between September 2003 and September 2006 (Table 1). Twelve adult patients referred to the Severe and Refractory Disease Unit, Dental Research Institute, University of Toronto, were considered to have RAP based on a specific set of criteria. Patients were deemed refractory to treatment and invited to participate in the study if they had been under the care of a periodontist for ≥1 year prior to their referral to the Severe and Refractory Disease Unit and demonstrated progressive attachment loss, despite adequate maintenance therapy. Each patient must have demonstrated attachment loss >2 mm in three or more sites while being monitored at our clinic over the course of a year. In all cases, a documented history of surgical treatment and/or antimicrobial therapy was noted. Patients also must have received regular subgingival scaling and prophylaxis every 3 months. Minimal amounts of microbial deposits were

Table 1.

Study Participant Demographics

Patient Group	Patients (N)	Mean Age (years)	Age Range (years)	Smokers (%)	Females (N)	Males (N)
RAP	12	45.83	30 to 58	0	6	6
CP	10	49	36 to 64	30	7	3
HC	13	33.25	28 to 45	0	6	7

present. The quantitative assessments of microbial deposits were inconsistent with the level of attachment loss observed. These patients were compared to 10 patients with a diagnosis of moderate to severe CP who responded positively to periodontal therapy and were deemed to be "periodontally stable," with no sites demonstrating attachment loss >2 mm over a 1-year period. These patients were obtained from the periodontal maintenance program at the Graduate Periodontal Clinic at the University of Toronto. Thirteen periodontally healthy subjects with no clinical signs of periodontal disease were used as controls for the study. All study participants were in good general health, with no underlying systemic diseases, and were not taking any medications. Participants were asked to donate 10 ml blood via venipuncture. This was a non-fasting blood sample. This study was approved by the Scientific and Ethics Review Boards at the University of Toronto. Informed consent was obtained from patients for all evaluations and procedures described in this report. The investigation was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Blood Neutrophil Isolation

A 10-ml blood sample was drawn into an EDTA-containing vacutainer.‡ Neutrophils were isolated using a one-step neutrophil isolation solution.§ Briefly, an aliquot of the blood was layered carefully over 3.5 ml of this solution (polymorphprep; sodium metrizoate, 13.8% [weight/volume (wt/vol)]; and dextran 500, 8.0% [wt/vol]), and the mixture was centrifuged at 1,600 revolutions per minute (rpm) for 30 minutes at 4°C. The neutrophils were harvested from the lower of two bands and washed by centrifugation‖ with Hanks balanced salt solution¶ at 2,500 rpm for 5 minutes. Any remaining red blood cells were lysed with 1 ml distilled water, and a neutrophil-rich pellet was obtained by centrifugation at 2,500 rpm for 5 minutes. The cells were resuspended in 1 ml Hanks balanced salt solution. A cell count was obtained with a hemocytometer. This method yields neutrophils with >90% cell viability as determined by trypan blue staining.

Neutrophil Functional Assays

Each day that a blood sample from a refractory or chronic periodontitis patient was acquired, a blood sample from a periodontally healthy control also was obtained and the following assays were performed.

Measurement of NADPH Oxidase Activity

Measurement of NADPH oxidase activity of the control and periodontitis patients was carried out using a protocol described previously.[17] Briefly, a 1×10^6 peripheral neutrophil suspension was incubated at 37°C for 15 minutes while shaking with 100 μl of a micromolar per liter concentration of dihydrorhodamine 123 (DHR)# dissolved in phosphate buffered saline (PBS).** The suspension was stimulated with 1 μl PMA†† at a working concentration of 10^{-5} M at 37°C for 10 minutes. Dihydrorhodamine oxidation to rhodamine by the respiratory burst of the cell was measured by flow cytometry.‡‡ The results are reported as the ratio of the mean channel fluorescence of stimulated cells (incubated with PMA and DHR) versus unstimulated cells (incubated with DHR only). This is expressed as the percentage increase over baseline.

sRBC Preparation

For assessment of Fc-gamma (Fcγ) receptor–induced phagocytosis, sheep erythrocytes (sRBCs)§§ were washed with Ca^{2+}- and Mg^{2+}-free PBS‖‖ and then opsonized with rabbit immunoglobulin G (IgG)¶¶ diluted 1:2,500 for 1 hour at 37°C. For assessment of complement receptor–induced phagocytosis, washed sRBCs were opsonized with mouse immunoglobulin M## diluted 1:50 for 1 hour at 37°C and then with complement C5–deficient human serum*** for 30 minutes at 37°C. Opsonized sRBCs were washed, labeled with Texas Red–sulfonyl chloride dye††† in a dilution of 1:5 for 20 minutes at 4°C, and rinsed well.

Phagocytosis With sRBCs

Prepared sRBCs and the isolated peripheral neutrophils were mixed together in the ratio of 5:1 and incubated for 25 minutes at 37°C in PBS.‡‡‡ For Fc receptor–induced phagocytosis, Alexa488-labeled monoclonal goat anti-rabbit secondary antibodies§§§ in a dilution of 1:500 were introduced to the mixture of neutrophils and IgG-coated sRBCs and incubated for 40 minutes at 4°C; for the complement receptor–induced phagocytosis, Alexa488-labeled monoclonal goat anti-mouse secondary antibodies‖‖‖ in a dilution of 1:500 were introduced and incubated in the same manner. The percentage of neutrophils positive for phagocytosis was determined by counting the number of neutrophils that had ingested at least one prepared sRBC in 200 neutrophils per condition with a fluorescence microscope.¶¶¶ This was expressed as a phagocytosis index.

‡ EDTA Vacutainer, Becton Dickinson, Rutherford, NJ.
§ Axis-Shield PoC, Oslo, Norway.
‖ Hettich Rotina 35R, Rare Scientific, Edmonton, AB.
¶ University of Toronto Media Preparation Services, Toronto, ON.
# Sigma Chemical, Burlington, ON.
** University of Toronto Media Preparation Services.
†† Sigma Chemical.
‡‡ Guava PCA, Guava Technologies, Hayward, CA.
§§ ICN/Cappel, Aurora, OH.
‖‖ University of Toronto Media Preparation Services.
¶¶ ICN/Cappel.
Cedarlane, Hornby, ON.
*** Sigma-Aldrich Canada, Oakville, ON.
††† Molecular Probes, Eugene, OR.
‡‡‡ University of Toronto Media Preparation Services.
§§§ University of Toronto Media Preparation Services.
‖‖‖ Molecular Probes.
¶¶¶ Eclipse CF160, Nikon, Mississauga, ON.

Assay Reproducibility

To validate the reproducibility and reliability of the sample collection, preparation, and analysis from day to day, three subjects were asked to donate 10 ml of blood on 2 consecutive days. Each functional assay was performed in the previously described manner on each control sample and then repeated the following day to verify the consistency of the results obtained. A Student *t* test for paired comparisons showed that the PMA-mediated mean oxygen radical production and the percentage of PMNs that phagocytosed the sRBCs via the Fcγ and complement receptors, from neutrophils taken from the same patient, yielded no significant difference when tested on 2 consecutive days. This confirmed that our assay methods were reproducible from day to day, enabling us to compare patient samples collected and tested on different days.

Statistical Analysis

The statistical analysis for the oxidase activity data was completed using single-factor analysis of variance (ANOVA) to determine whether a difference existed between the three groups. Student *t* tests were used to determine the significance of the difference between the RAP group and the CP and HC groups. To assess the magnitude of the difference in mean oxygen radical production between the RAP group and the CP group, values for these groups were calculated as a percentage of the periodontally HCs tested on the same day and compared to 100%.

The phagocytosis data also did not fit a normal distribution curve. Therefore, prior to one-way ANOVA, standard transformations were executed on the data by taking the arcsine of the square root of the original value.

RESULTS

NADPH Oxidase Activity as Measured by DHR

Neutrophils from RAP patients generated significantly (P <0.03) higher levels of oxygen radicals as measured by dihydrorhodamine conversion to rhodamine123 compared to periodontally HCs after direct stimulation of intracellular protein kinase C (PKC) with PMA. Neutrophils from CP patients generated significantly fewer (P <0.006) oxygen radicals compared to periodontally HCs after stimulation with PMA (Fig. 1). The values used to measure the increase in oxygen radical production were calculated by taking the ratio of mean channel fluorescence of cells loaded with DHR and PMA (stimulated) divided by the mean channel fluorescence of cells loaded with DHR only (baseline). Of note, 11 of the 12 RAP patients produced higher mean levels of oxygen radicals compared to the HC used that same day. All 10 of the CP patients produced lower mean levels of oxygen radicals compared to the HC used that same day. To assess the magnitude and significance of the difference in oxygen radical production between the RAP and CP groups, the patient values were expressed as a percentage of the value of the HC tested on the same day. The generation of oxygen radicals as measured with flow cytometry was an average of 236% higher for the RAP group and an average of 44% lower for the CP group compared to the HC of that day (P <0.001; Fig. 2).

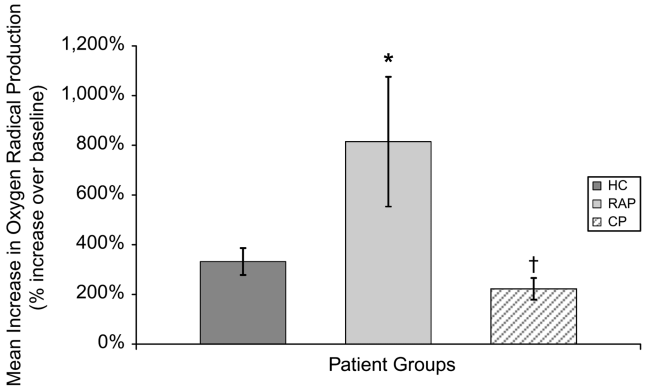

Figure 1.
*Mean increase in oxygen radical production over baseline in the RAP, CP, and HC groups after stimulation with PMA. A significant difference existed between the groups (ANOVA; P <0.01). Error bars represent the SEM. *The RAP group produced a higher mean increase in oxygen radical production compared to the HC group (Student t test; P <0.03). †The CP group produced a lower mean increase in oxygen radical production compared to the HC group (Student t test; P <0.006).*

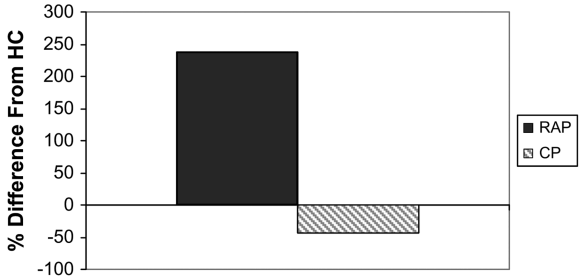

Figure 2.
The magnitude of the difference in oxygen radical production of the RAP and CP groups compared to HC tested the same day. The difference was significant between the two groups (Student t test: P <0.004).

Neutrophil Phagocytosis

There was a significant increase in neutrophil phagocytosis of sRBCs after complement receptor activation in RAP subjects compared to CP and HC groups (P <0.05; Fig. 3). Although there was a trend that

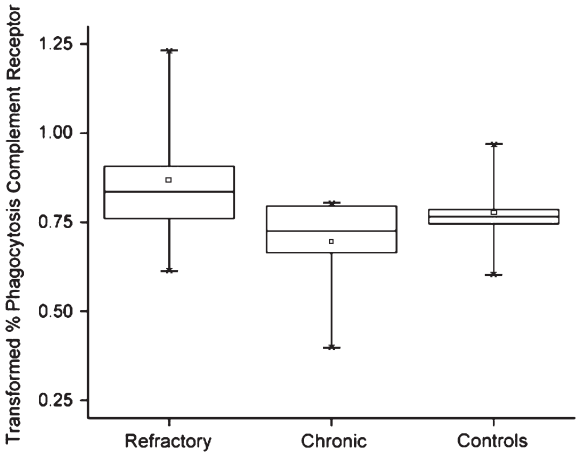

Figure 3.
Boxplot of the percentage of PMNs that phagocytosed the prepared sRBC from the RAP, CP, and HC groups via the complement receptor. The bars across the boxes are the medians of the distributions, whereas small squares mark the means. The boxes delineate the 25th percentile to the 75th percentile of each group. Dashes at the ends of the whiskers mark the minima and maxima (which are also the 5th and 95th percentiles in this case). A significant difference was noted between the RAP and the CP groups (P = 0.047).

the neutrophils derived from RAP patients phagocytosed more sRBCs after Fc receptor activation compared to the CP group, the results were not significant ($P = 0.078$).

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate hyperactive NADPH oxidase activity in activated neutrophils in patients with RAP compared to periodontally HCs and patients with successfully treated CP. NADPH oxidase activity was measured using DHR and flow cytometry. Most of the studies examining the oxidative burst of peripheral PMNs use luminol-enhanced chemiluminescence, a well-established method of determining the extent of oxygen free-radical release.[18] However, microcytofluorometry using DHR also has been shown to be a fast, easy, and reliable method for evaluation of the production of reactive oxygen intermediates from peripheral PMNs.[19]

Upon activation of the neutrophils with PMA, nonfluorescent DHR is oxidized to its fluorescent derivative, rhodamine 123, by the H_2O_2 generated by the activated NADPH oxidase.[20] A wide variety of soluble particles and molecules can activate the NADPH oxidase membrane complex found on the membranes of neutrophils and mononuclear phagocytes.[21,22] It is this membrane complex that is responsible for the generation of reactive oxygen species, including superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet molecular oxygen.[23] Not only are these reactive oxygen metabolites important antibacterial products of the neutrophils, they also may have the potential to initiate tissue damage that could lead to the localized breakdown of periodontal tissues.[24] In fact, increased levels of oral neutrophils, as measured with an oral rinse, correlated positively with periodontal disease severity,[25] suggesting that the presence of neutrophils and their byproducts in the gingival crevice are partly, if not wholly, responsible for the destruction of periodontal tissues in various forms of periodontal disease.

In this study, PMA was used to stimulate the neutrophils. Although PMA is a laboratory tool that is not related to any physiological stimulus that PMNs may encounter in vivo, it is very useful for assessing in vitro neutrophil functions, including the generation of reactive oxygen species. This soluble phorbol ester diffuses through the cell membrane to directly activate PKC. In normal healthy neutrophils, a phosphatidylinositide pathway initiated by phospholipase C and a phosphatidylinositide pathway initiated by phospholipase D result in the activation of PKC via diacylglycerol and the formation of myoinositol-1,4,5-triphosphate.[9] PKC then phosphorylates several downstream signaling proteins, resulting in events such as chemotaxis, phagocytosis, respiratory burst, and lysosomal enzyme release. By using PMA, the activation of the cells is not based on a receptor ligand–mediated response. This approach suggests that the neutrophils from the RAP patients have an intrinsic hyperreactive pathway from PKC to the NADPH oxidase complex that does not seem to be related to quantitative or qualitative differences in membrane receptors.

In previous studies of patients with CP, increased oxygen radical production was evident compared to periodontally HCs only when neutrophils were activated via the Fcγ receptor pathway.[6,11] It was concluded that this difference may be due to a constitutionally greater responsiveness of the Fc receptor of the neutrophils in these patients.[11] These results seem to indicate that in CP patients there may be an intrinsic aberrant signaling pathway linked to the Fcγ receptor that is causing the increased production of oxygen free radicals. This result was supported by our observations in that there was no significant increase in oxygen radical production evident in the CP group after stimulation with PMA. Although we did detect a slight decrease in PMA-induced oxygen radical production in the CP group compared to HCs, this may be a result of the successful periodontal treatment and decreased bacterial load in this population immediately following therapy. The striking difference in our study was that in the unique group of RAP patients, PMA-induced oxygen radical production was significantly

greater than that of the CP group and HCs, a result that was not found in previous studies of patients with CP. Further studies will be required to determine the exact mechanism of the hyperactive phenotype and whether RAP patients have membrane-based signaling defects as well.

Neutrophils from the RAP group showed increased mean oxygen radical production and increased phagocytotic activity when activated via the complement receptor. It seems that the neutrophils isolated from these patients are essentially hyperreacting compared to their healthy counterparts. The neutrophils are able to produce more reactive oxygen species and phagocytose more bacteria. This hyperreactivity may be part of the hyperactive neutrophil phenotype that results in the continued periodontal breakdown observed in these patients. It also is possible that this observed PMN hyperreactivity is due to priming as a result of the inflammation associated with the periodontal disease or exposure to the pathogenic bacteria associated with periodontitis. This is significant because this priming is further evidence that a localized chronic oral infection may impact the systemic innate immune response.

This study was carried out using a unique population of patients presenting to the Severe and Refractory Diseases Unit at the University of Toronto. Although the "refractory periodontitis" disease category was eliminated as a separate disease entity in 1999 at the International Workshop for a Classification of Periodontal Diseases and Conditions,[26] it was concluded that the term "refractory" could be applied to all forms of periodontal disease in the new classification system (e.g., refractory chronic periodontitis and refractory aggressive periodontitis) because a small percentage of cases of all forms of periodontitis might be unresponsive to treatment. We attempted to avoid confounding factors by ensuring that all patients were in good health and were not taking medications. We also attempted to examine patients in all groups who were non-smokers; however, three patients in the CP group did report a smoking habit of <10 cigarettes a day. The neutrophil functions in these three patients were not significantly different from the other control patients (data not shown). In a study by MacFarlane et al.,[27] neutrophil function was assessed in a group of refractory periodontitis patients; phagocytosis was significantly impaired in the refractory group compared to periodontally HCs. However, the striking difference in their study population compared to ours was that 90% of their refractory subjects were smokers. We removed cigarette smoking as a confounding variable in our refractory group because smoking adversely affects the chemotactic and phagocytic ability of normal neutrophils, resulting in compromised neutrophil functions.[28]

Although attempts were made to match the HCs in terms of age and gender to the refractory or chronic subjects tested on a specific day, it was not always feasible because of the younger population of HCs that were available to participate in the study. Although the mean age of the HCs was significantly lower than that of the two disease groups, we observed neutrophil hyperactivity in the older refractory disease group. This is in contrast to previous studies that demonstrated that neutrophil functions, such as chemotaxis, and superoxide anion production decrease with age.[28,29] However, some functions, including phagocytosis, consistently have been found not to change with aging.[30] Although the mean age of the refractory patients in our study was higher than that of the HCs, they still exhibited neutrophil hyperactivity, thereby leading to the suggestion that age was not a factor related to the increases in oxygen radical production and phagocytotic activity observed in this study.

CONCLUSIONS

The larger respiratory burst and higher phagocytotic activity observed in neutrophils derived from patients with RAP, compared to neutrophils derived from CP patients and periodontally HCs, might account for the continued periodontal breakdown, despite ongoing periodontal therapy. The mechanism that results in this neutrophil hyperactivity in this unique patient population remains to be determined.

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