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ENHANCED SUPEROXIDE PRODUCTION WITH NO CHANGE OF THE ANTIOXIDANT ACTIVITY IN GINGIVAL FLUID OF PATIENTS WITH CHRONIC ADULT PERIODONTITIS

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In the gingival crevicular fluid (GCF) of control and chronic adult periodontitis (CAP) patients there is a spontaneous release of $O_2^{\cdot-}$ radicals from polymorphonuclear leukocytes (PMN). The addition of the exogenous stimuli phorbol myrystate acetate (PMA) decreased the $O_2^{\cdot-}$ formation in control GCF, while in CAP patients produced a marked enhancement of $O_2^{\cdot-}$ generation.

The circulating PMN of control subjects did not show a spontaneous $O_2^{\cdot-}$ formation, differently from CAP patients. On the contrary, a similar $O_2^{\cdot-}$ production was measured when the circulating PMN were stimulated with PMA.

Moreover, the antioxidant activity measured in 10 µl of cell free gingival supernatant (GS) of control and CAP patients had the same values by inhibiting 12.6% and 18.9% respectively of the $O_2^{\cdot-}$ formation supported by a xanthine/xanthine oxidase system.

Probably, the protective or destructive effect of PMN in GCF of CAP patients depends on the variations of the rate of $O_2^{\cdot-}$ formation in respect to the intrinsic antioxidant property of GS.

KEY WORDS: gingival fluid, polymorphonuclear leukocytes, superoxide, superoxide dismutase, chronic adult periodontitis.

ABBREVIATIONS: PMN, polymorphonuclear leukocytes; GCF, gingival crevicular fluid, $O_2^{\cdot-}$, superoxide, CAP, chronic adult periodontitis; PMA, phorbol myrystate acetate; GS, cell free gingival supernatant.

INTRODUCTION

Several lines of evidences implicate the polymorphonuclear leukocytes (PMN) as major protective cells against oral bacterial pathogens.[1]

The PMN are present in the gingival crevicular fluid (GCF) and their level increases at sites of gingival inflammation.[2] Interaction between bacteria and PMN activates a complex series of biochemical events, resulting in the killing of the microorganisms.[3] During phagocitosis, the crevicular PMN releases potent biologic components into GCF, such as derivates of arachidonic acid,[4] lysosomal enzymes[5] and antimicrobic substances such as lactoferrin and lysozyme.[6] These events, if not adequately controlled or neutralized, can contribute to periodontal tissue destruction by long standing PMN stimulation. However, the early biochemical process in the activation

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of the PMN is the stimulation of NADPH oxidase, an enzyme which produces a flux of superoxide anions ($O_2^{\dot{-}}$), initially direct to the extracellular space.[7] These radicals formed by PMN, together with their derivates (H_2O_2; hydroxyl radical $OH^{\cdot}$), are highly toxic.[8]

They initiate the bacterial destroying process; however, when these radicals are not limited into phagocitic vacuoles they can also react with several biological compounds leading to extracellular structure degradation.[9]

Therefore, several factors and humoral conditions can decide if the response of PMN to gingival microorganisms is protective or destructive (entity of PMN intrinsic activation, quantity and quality of stimuli), but certainly, one of the most important factors is the ability of the GCF to neutralize the excess of oxy-radicals. To our knowledge, no data are available up to now, about the oxy-scavenger properties of GCF. The present study was undertaken to determine the relationship between the $O_2^{\dot{-}}$ generating activity of crevicular PMN and the antioxy-radical ability of GCF from healthy and chronic adult periodontitis (CAP) patients.

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MATERIALS AND METHODS

Materials

Hanks' balanced salt solution (Hank's BBS) was purchased from GIBCO (Grand Island, NY). Cytochrome *c* (type VI from horse heart), superoxide dismutase (bovine erythrocytes; 3000 units/mg prot), phorbol myrystate acetate (PMA) and xanthine oxidase (2 units/mg prot) were purchased from Sigma Chemical Co (St. Louis, Mo). All other reagents were from Merck (Darmstad, W. Germany).

Patient selection

This study included: 16 healthy volunteers subjects (control) with no clinical evidence of gingivitis (gingival index close to O[10]) and no radiographic signs of bone loss and receiving no drugs; mean age was 25 yr with a range of 23–27 yr.

14 CAP patients with radiographic evidence of generalized alveolar bone loss and probable pocket depths in excess of 4 mm at more than 4 sites; mean age was 54.9 yr with a range of 49–62 yr.

GCF collection and processing

GCF was collected by washing the gingival crevices 12 times with 10 μl of Hanks' BBS containing 5 mM glucose by using a sterile microsyringe as described by Skapski and Lehner.[11] To avoid contamination, the sampling area was isolated with cotton rolls and the saliva aspirated. In the CAP patients, the GCF was collected where unequivocal signs of chronic adult periodontitis were evident. 5 to 10 maxillary buccal interdental areas were washed per individual, resulting in a pooled collected volume of 120–150 μl, which was stored in ice. 20 microliters were immediately used for PMN count.

The measuring of PMN was done by a Coulter mod. S cell counter after diluting the GCF with the medium as described by Kubitschek.[12] Cell free gingival washing supernatants (GS) were obtained by centrifuging 50 μl of GCF in a microcentrifuge at 400 g for 5 min. The GS were kept frozen in aliquots at -20°C until use.

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Isolation of blood PMNs

The circulating PMN were prepared from heparinized blood taken from control of CAP patients by utilizing a combined dextran/Ficoll-Hypaque separation procedure and hypotonic lysis to remove contaminating erythrocytes.[13] The resulting cells (96–98% neutrophils; 2–4% monocytes) were washed twice in phosphate buffer containing 125 mM NaCl; 8 mM Na_2HPO_4; 2 mM NaH_2PO_4; 5 mm KCl, 5 mM glucose; pH 7.4 and finally suspended in 200 μl of the same buffer.

Superoxide generation assay

The release of $O_2^{\dot{-}}$ by isolated blood PMN or by crevicular PMN was determined in duplicate as previously described[13] by superoxide dismutase-inhibitable reduction of cytochrome *c*. The incubation mixture (total volume 800 μl) contained: 75 μM cytochrome *c* and 5×10^6 PMN, or 30 μl GCF in Dulbecco buffer (138 mM NaCl; 2.7 mM KCl; 16.2 mM Na_2HPO_4; 1.47 mM KH_2PO_2; pH 7.4) containing 5 mM glucose. After 1 min incubation, the reduction of ferricytochrome *c* was followed at 550 nm in a Perkin Elmer 559 dual-beam spectrophotometer with the cuvette holder thermostated at 37°C (spontaneous $O_2^{\dot{-}}$ generation). After 3 min, 0.1 μg/ml PMA was added to the incubation mixture and the reaction was monitored in the following 3 min (stimulated $O_2^{\dot{-}}$ generation). Reference cuvette contained 10 μg/ml superoxide dismutase. $O_2^{\dot{-}}$ generation was calculated by using an absorbance coefficient of 21.1 $mM^{-1}cm^{-1}$ for reduced cytochrome *c*.

GS antioxidant activity

The assay used the xanthine oxidase/xanthine reaction as source of $O_2^{\dot{-}}$, with cytochrome *c* as the indicating scavenger for this radical. The incubation mixture contained: 100 mM potassium-phosphate buffer; 0.5 mM xanthine; 0.35 μM ferricytochrome *c* and enough xanthine oxidase (6 μM) to cause $A_{550} = 0.025$/min at pH 7.8 and 25°C, in a total reaction volume of 800 μl. This system produced 0.84 nmol $O_2^{\dot{-}}$/min. After 1 min, 10 μl of GS were added and the reduction of cytochrome *c* was monitored for the following 2 min. The GS scavenger activity was determined by evaluating the percent difference in the slopes of cytochrome *c* reduction before and after GS addition and considering that 10 μg superoxide dismutase caused a 90% inhibition.

Statistical analysis

The results are the mean $\pm$ S.D. A one way ANOVA, followed by Duncan's multiple range test was performed on all the data. Differences were considered significant when $p < 0.05$.

RESULTS

Table I shows the rate of $O_2^{\dot{-}}$ formation by PMN contained in GCF of control and CAP patients. In both groups of patients, the crevicular PMN released $O_2^{\dot{-}}$ spontaneously, with no statistical difference between the two groups. After PMA stimulation, the production of $O_2^{\dot{-}}$ was significantly lower in control patients, while in

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TABLE I
$O_2^{\cdot-}$ generation in crevicular fluids of control and CAP patients

	PMN count (number/10 μl GCF)	pmol $O_2^{\cdot-}$/min PMN	
		Spontaneous	Stimulated
Control (n = 16)	77.5 ± 9.8	9.25 ± 3.72	8.13 ± 1.86[b]
CAP (n = 14)	103.8 ± 9.0	6.60 ± 2.30	11.64 ± 4.30[a,b]

The formation of $O_2^{\cdot-}$ was monitored by following the superoxide dismutase-inhibitable reduction of cytochrome *c* in the absence (spontaneous) or in presence of PMA (stimulated).
The results are mean ± S.D.
[a]$p < 0.05$, significant difference from the corresponding control group; $p < 0.05$, significant difference from the corresponding spontaneous group.

TABLE II
Formation of $O_2^{\cdot-}$ by PMN isolated from circulating blood of control and CAP patients

	nmol $O_2^{\cdot-}$/min PMN	
	Spontaneous	Stimulated
Control (n = 16)	0	8.8 ± 0.07[b]
CAP (n = 14)	0.45 ± 0.08[a]	6.3 ± 0.08[b]

5×10^6 PMN suspended in phosphate buffer were used to monitor $O_2^{\cdot+}$ generation in absence (spontaneous) or in presence of PMA (stimulated).
[a]$p < 0.05$, significant difference from the corresponding group.
[b]$p < 0.05$, significant difference from the corresponding spontaneous group.

the CAP patients $O_2^{\cdot-}$ generation was markedly enhanced in comparison to both spontaneous $O_2^{\cdot-}$ formation and control stimulated $O_2^{\cdot-}$ production. The same table shows that the number of PMN is significantly enhanced in CAP patients with respect to control.

Table II reports the production of $O_2^{\cdot-}$ from PMN isolated from blood of control and CAP patients. The spontaneous $O_2^{\cdot-}$ formation was evident only in the circulating PMN of CAP patients, whereas similar not significant values of stimulated $O_2^{\cdot-}$ production were evident in control and CAP patients after PMA stimulation.

Table III shows that crevicular GS contained an antioxidant activity able to scavenger the $O_2^{\cdot-}$ radicals which did not differ between control and CAP patients.

TABLE III
Antioxidant activity of GS of control and CAP patients

	% Inhibition 10 μl GS	ng SOD equivalent/ 10 μl GS
Control (n = 16)	12.6 ± 3.1	13.2 ± 3.5
CAP (n = 14)	18.9 ± 3.8	20.4 ± 4.0

The antioxidant activity was measured by evaluating the scavenger property of 10 μl GS added to a xanthine/xanthine oxidase – $O_2^{\cdot-}$ generating system.
The results are means ± S.D.

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The values are reported as % inhibition of the xanthine/xanthine oxidase $O_2^{\cdot-}$ generating reaction, or in equivalents of ng SOD present in 10 μl of GS.

DISCUSSION

The present study indicates that in the GCF there is a spontaneous production of $O_2^{\cdot-}$ by crevicular PMN, both in control and in CAP patients. The ability of the crevicular PMN to release $O_2^{\cdot-}$ was fully expressed in control subjects, differently from CAP patients where it was necessary to add the exogenous stimulus PMA to obtain maximal PMN activation. This behaviour could be caused by some compounds which inhibit part of the PMN activation in the gingival fluid of CAP patients. Therefore, only in the presence of PMA all the crevicular PMN became active for the $O_2^{\cdot-}$ generation, due to a potent stimulus which activates the NADPH oxidase enzyme at post receptor sites.[7] We cannot exclude that these results can be caused by the different ages between control and CAP patients, even if preliminary results obtained on control subjects of 50–55 yrs seem to exclude this possibility. The concept of a latent PMN inhibitor of GCF patients has previously been suggested by Murray and Patters[14] who demonstrated that factors released from plaque microorganisms reduce PMN function. We can speculate that, if the inhibitory mechanisms reducing PMN oxidative burst in the CAP patients are removed, probably in the GCF of these patients, a higher level of $O_2^{\cdot-}$ could be dangerous and responsible for the activity of the disease.[15] The spontaneous $O_2^{\cdot-}$ formation in GCF could be caused by the contact of crevicular PMN with endogeneous factors present in the gingival sulcus.[16,17] In fact, the circulating PMN of control subjects did not show a spontaneous $O_2^{\cdot-}$ generation, but this property is evident when the PMN are present in the sulcus. On the contrary, the circulating PMN of CAP patients show a spontaneous $O_2^{\cdot-}$ formation which could be considered an index of PMN activation, probably produced by the release of endogeneous effector from the inflammed gingival tissue. This hypotesis has in part been confirmed recently by Zafiropoulos *et al.*[18] who found in plasma of patients with periodontal disease the presence of products released from degranulated active PMN. The present research also demonstrates that in the GCF there is an oxy-scavenger activity (anti-oxidant). A macromolecular origin of this inhibitory activity is suggested because it was lost after exposition of GCF to heat denaturation (data not shown). It is likely that superoxide dismutase could be responsible for the $O_2^{\cdot-}$ scavenger ability present in GCF because a detectable amount of this enzyme is found in extracellular fluids.[19] Another extracellular macromolcule such as ceruplasmin can be considered an efficient oxy-scavenger.[20] Probably, an appropriate balance between the state of activation of crevicular PMN and the antioxidant activity of GCF is a critical condition which favours a protective function of PMN for periodontium. If a full crevicular PMN activation is present concomitantly with an elevation of their concentration and with no change or reduction of the GS antioxidant activity, probably the gingival tissues can be injured by $O_2^{\cdot-}$ mediated reactions. This last situation could occur in the GCF of CAP patients where there is an increased concentration of PMN in comparison to controls[21] with not difference in the antioxidant activity of gingival fluid.

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