Effect of bacterial stimulants on release of reactive oxygen metabolites from peripheral blood neutrophils in periodontitis

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Key words: *periodontitis, neutrophils, luminol, chemiluminescence.*

Summary. *The aim of the present investigation was to explore the oxidative activity of peripheral blood polymorphonuclear neutrophils of periodontitis patients and of healthy subjects stimulated with non-opsonized E. coli and lipopolysaccharide of E. coli.*

Material and methods. The leukocytes for this study were obtained from peripheral venous blood of 22 parodontitis patients and 16 healthy subjects. Oxidative activity of peripheral blood polymorphonuclear neutrophils was measured by method of the luminol-dependent chemiluminescence.

Results. The luminol-dependent chemiluminescence of stimulated neutrophils of periodontitis patients with non-opsonized E. coli increased less significantly ($p<0.001$) as compared to analogous chemiluminescence of control subjects (147126±8386 cpm and 189247±9134 cpm, respectively). However, the luminol-dependent chemiluminescence of stimulated neutrophils of periodontitis patients with lipopolysaccharide was five times higher than that of the subjects with intact periodontal tissues and comprised 13261±1251 cpm and 2627±638 cpm, respectively.

Conclusions. Our study results show a complex dependence of oxidative function of peripheral polymorphonuclear neutrophils of periodontitis patients upon the nature of stimulants. Therefore further attempts should be made to evaluate its significance in the etiopathogenesis of periodontal tissue diseases of inflammatory origin.

Introduction

Disease of periodontal tissues is considered to be an inflammatory disorder, pathophysiology of which is related to accumulated dental microbial plaque and the host response to this accumulation (1–3). The host reaction to gingival microorganisms is characterized in part by an influx of polymorphonuclear leukocytes (PMN), which is one of the most important steps in host defense (4).

Neutrophils are the predominant leukocytes in the gingival pocket epithelium and the adjacent connective tissue (5). They play a protective role in the periodontium and patients with severe quantitative or qualitative defects of their function may be predisposed to periodontal diseases (6). The accumulation of these cells at the sites of inflammation is accompanied by modification of their activity/ability to release granule content and toxic oxygen radicals (7).

The oxygen metabolites are thought to be produced both at the plasma membrane and the phagolysosomal membrane and consequently released into phagolysosomes and the extracellular environment (8). It was shown that the primary oxygen species produced by leukocytes and other phagocytes are superoxide ions (9). Superoxide ion may be released by the cells or be converted into much more active oxygen species such as hydroxyl or hydroxyl-like free radicals (10) where the superoxide-driven Fenton reaction (reactions 1 and 2) and the Haber-Weiss reaction (reaction 3) occur:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (2)$$

$$H_2O_2 + O_2^- \rightarrow OH^- + \cdot OH \quad (3)$$

Indeed, release of hydroxyl radicals by stimulated neutrophils, monocytes and macrophages has been shown (11). Oxygen radicals are bactericidal and fungicidal. Extracellular bacteria can also be killed but principally by O_2-dependent mechanisms (12). They also play an important role in neutrophil-related tissue injuries, as well as oxygen-independent cytotoxic mechanisms including elastase, cathepsin G, collagenase, lipases, phospholipases, bactericidal/permeability-increasing protein, defensin and lactoferrin (13).

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There is a clearly defined substantial role for reactive oxygen metabolites in periodontitis (9, 14), however little research has been performed in this area (14).

The yields of superoxide ion and other oxygen species can be measured by means of the luminol-dependent chemiluminescence (CL) (15, 16).

It is known that the intensity of PMN chemiluminescence depends on the nature and concentration of substances stimulating these cells (17). For PMN stimulation chemical materials, opsonized microorganisms, and lipopolysaccharides (LPS) were mostly used. In medical literature we found no findings concerning studies of the luminol-dependent CL of peripheral blood neutrophils from periodontitis patients stimulated by non-opsonized microorganisms. It is possible that in the gingival crevice environment PMN contact with non-opsonized microorganisms and their toxins. Hence, the response to non-opsonized microorganisms and their toxins can play a decisive role in development of periodontitis.

The aim of the present investigation was to explore oxidative activity of peripheral blood polymorphonuclear neutrophils from periodontitis patients and healthy subjects stimulated with non-opsonized *E. coli* and lipopolysaccharide of *E. coli*.

Material and methods

Study patients were selected from a large number of individuals with pathology of periodontal tissues who were examined clinically and radiographically and were diagnosed as having periodontitis. They underwent periodontal treatment or routine check-ups at the Department of Odontology of Kaunas University of Medicine Hospital. We chose and included in our study only the patients with very marked signs of periodontitis using A. L. Russell's (18) periodontal index (PI) (Table 1). The study was performed on 38 systemically healthy subjects aged 18–50 years: 22 patients (9 males and 13 females) with periodontitis and 16 (7 males and 9 females) periodontally healthy controls, chosen from donors, dental students and personnel.

Composition of study groups. Control group (with healthy periodontal tissues) comprised persons having PI not exceeding 0.0 point, and periodontitis group was composed of subjects whose PI was 4.1–8.0 points.

Laboratory studies. Ten milliliters of venous blood were collected from the subjects by venipuncture and anticoagulated with heparin (20 u/ml).

The test tubes with blood were positioned at the angle of 45 degrees and kept for 1 h at 37°C. Then the supernatant plasma, rich in leukocytes, was aspirated, mixed and taken in portions of 1 ml in the vials at 37°C. The intensity of spontaneous cellular CL to an amplifier (luminol) was measured. After 5 min, the 0.1 ml suspension of non-opsonized *E. coli* and LPS of *E. coli* in Hank's balanced salt solution (HBSS) was added and the light reaction was followed after 15, 30, 45, 60 and 75 min. The light intensity was registered by a liquid scintillation counter "Delta-300" at the Department of Biochemistry of Kaunas University of Medicine.

It is known that PMN make a major contribution to the total CL response of whole blood or isolated cell suspensions (19). Therefore, the intensity of non-stimulated and stimulated CL depends linearly on the amount of PMN in cell suspensions. Because of this, the CL intensity induced by PMN ($I_{(PMN)}$) can be calculated from the CL intensity of the total leukocyte fraction ($I_{(leuk)}$) using equation (20):

$$I_{(PMN)} = I_{(leuk)} \times 100\ V/vcn,$$

v is the cell suspension volume (ml), V – volume of the cuvette (ml), c – the leukocyte concentration and n – the PMN content (%).

Reagents. Luminol, HBSS and LPS were obtained from Sigma Chemical Co. (St. Louis Mo).

E. coli ATCC25922 was obtained from Microbiology Laboratory of Kaunas University of Medicine Hospital.

The final luminol concentration was 50 μM, LPS – 0.2 ng/ml. The final *E. coli* concentration was 6×10^6 cells/ml.

Statistical analysis. The significance of the differences between healthy controls and patients was evaluated by Student's (t) test.

Results

Clinical data. The mean age of the periodontitis group was 38.2±2.9 years and that of the control group subjects was 29.5±3.6 years. The difference in mean age between the groups was not significant ($p>0.05$).

Russell's PI in the control group subjects equaled 0, and in the periodontitis subjects it was 5.58±0.37 (Table 1).

Laboratory data. The results of measurements of the luminol-dependent CL of non-stimulated peripheral blood leukocytes of periodontitis patients and control group subjects are given in Table 2. The data confirm that basal levels of the luminol-dependent CL of blood leukocytes from periodontitis patients always exceed those of control group subjects ($p\leq0.001$). Non-opsonized *E. coli* and LPS significantly enhanced the

Table 1. **Investigation data of the subjects and clinical evaluation**

Investigation groups	n	Sex		Age (years)	Rusell's PI (points)
		females	males		
Periodontitis patients	22	13	9	38.2±2.9	5.58±0.37
Controls	16	9	7	29.5±3.6	0

Table 2. **Maximal luminol-dependent chemiluminescence of peripheral venous blood neutrophil leucocytes of investigation groups patients**

Investigation groups	n	1×10^6 NL chemiluminescence (cpm)		
		non-stimulated	After stimulation	
			non-opsonized *E. coli*	LPS
Periodontitis patients	22	381±50	147126±8386	13261±1251
Controls	16	135±13	189247±9134	2627±638
p		≤0.001	≤0.01	≤0.001

luminol-dependent CL induced by blood leukocytes of periodontitis patients and control group subjects ($p\leq0.001$). Interestingly, the luminol-dependent CL of blood leukocytes from control group subjects, stimulated by non-opsonized *E. coli*, exceeded analogous CL of periodontitis patients ($p\leq0.01$) (Table 2).

It should be noted that LPS also significantly enhanced the luminol-dependent CL of peripheral blood leukocytes both from periodontitis patients and control group subjects. However, the above-mentioned CL of periodontitis patients substantially ($p\leq0.001$) exceeded that of the control group subjects.

The development of the luminol-dependent CL of peripheral blood leukocytes from periodontitis patients and control group subjects stimulated by non-opsonized *E. coli* and LPS is presented in Figure. The data show that the luminol-dependent CL stimulated by LPS, reaches its maximum no longer than in 15 minutes; whereas the luminol-dependent CL of peripheral blood leukocytes both from periodontitis patients and control group subjects, stimulated by non-opsonized *E. coli*, reaches its maximum approximately in 1 hour.

Discussion

As has been shown by investigations in recent years, periodontal diseases are initiated by subgingival infection, but the presence of microorganisms alone is not the only factor responsible for periodontal destruction (21, 22). The responses of the host to pathogenic bacteria are thought to be critically important (2).

The determined clinical parameters (Table 1) showed significantly increased PI in group of periodontitis patients in comparison with the control group subjects. Bearing in mind that interactions between bacteria and the immune system play a central role in the etiology of periodontal disease (23, 24), it was expected that our selection of subjects with severe periodontal lesions of inflammatory origin in the periodontitis group would provide clear data on the interaction between leukocytes and bacteria and their toxins, as compared with analogous data for the control group subjects. Other authors (24, 25) have used a similar method for arrangement of study groups.

Our results of the luminol-dependent CL of peripheral neutrophil leucocytes (NL) of periodontitis patients stimulated with LPS always exceeded that of control subjects ever five times. These data coincide with those of L. Shapira et al (16) indicating that the preincubation of PMN from rapidly progressive periodontitis patients with LPS from the periopathogen, *Porphyromonas gingivalis*, primes PMN for enhanced O_2^- production, in contrast to PMN from a control group. Other authors (23, 26) state that the responsiveness to priming LPS on CL was slightly but not significantly higher in the periodontitis group.

The difference of study data obtained by some authors investigating CL might be explained by the fact that other methods were used for release of leukocytes. It is known that Ficoll procedure can cause cell activation (7).

We obtained interesting data on the luminol-dependent CL of peripheral blood leukocytes from periodontitis patients stimulated by non-opsonized *E. coli*, which was substantially ($p\leq0.01$) lower than that of

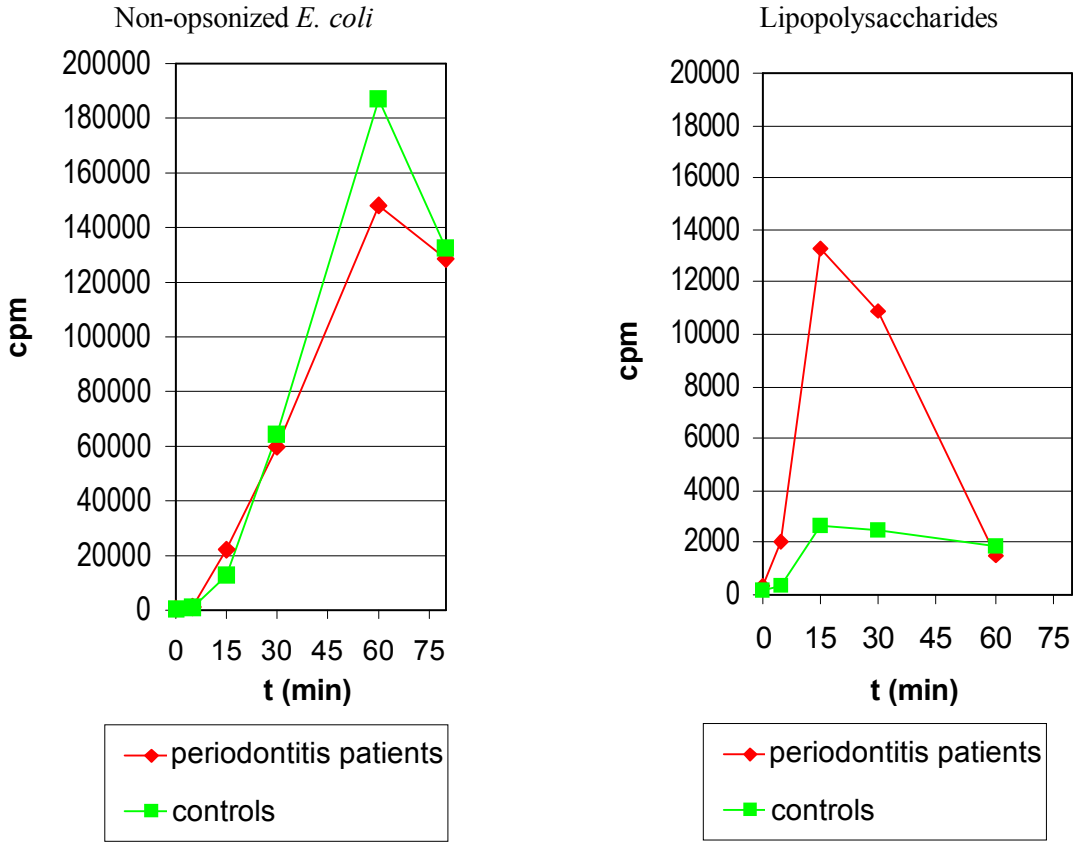

Fig. **Dependence of luminol-enhanced chemiluminescence upon the state of periodontal tissues and various bacterial stimuli**

the control group subjects. In medical literature we found no similar findings. Our data might indicate an insufficient protective function of periodontal tissues of periodontitis patients. For this reason microbes accumulate in the environment of periodontal tissues and their toxins (LPS) activate PMN of the gingival crevice. The activated PMN release a large amount of reactive oxygen metabolites, which promote tissue destruction.

These findings could explain the site-specific nature of periodontal diseases and factors in their progression.

Conclusions

Basal levels of the luminol-dependent chemiluminescence of peripheral blood leukocytes of periodontitis patients are substantially higher than those of the subjects with intact periodontal tissues. Non-opsonized *E. coli* and lipopolysaccharides enhanced the luminol-dependent chemiluminescence of peripheral blood leukocytes of periodontitis patients and control group subjects. The luminol-dependent chemiluminescence of peripheral blood neutrophils from periodontitis patients stimulated by non-opsonized *E. coli* is mainly ($p \leq 0.01$) lower than that of the control group subjects. The luminol-dependent chemiluminescence of peripheral blood neutrophils from periodontitis patients stimulated by lipopolysaccharide is even 5 times higher than that of the control group subjects.

Bakterinės kilmės aktyvatorių įtaka aktyviųjų deguonies formų išsiskyrimui iš periferinio kraujo neutrofilinių leukocitų sergant periodontitu

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Raktažodžiai: periodontitas, neutrofiliniai leukocitai, luminolas, chemiliuminescencija.

Santrauka. *Darbo tikslas.* Ištirti sergančiųjų periodontitu ir asmenų, kurių priedančio audiniai sveiki,

periferinio veninio kraujo neutrofilinių leukocitų, stimuliuotų neopsonizuotomis *E. coli* ir *E. coli* lipopolisacharidu, oksidacinę funkciją.

Medžiaga ir metodai. Ištirta 22 sergančiųjų periodontitu ir 16 kontrolinės grupės asmenų periferinio veninio kraujo neutrofiliniai leukocitai. Periferinio veninio kraujo neutrofilinių leukocitų oksidacinė funkcija buvo matuojama nuo luminolo priklausomos chemiliuminescencijos metodu.

Rezultatai. Neopsonizuotomis *E.coli* stimuliuotų sergančiųjų periodontitu neutrofilinių leukocitų nuo luminolo priklausoma chemiliuminescencija iš esmės buvo mažesnė ($p<0,01$) už analogišką asmenų, kurių priedančio audiniai sveiki, ir sudarė atitinkamai – 147126±8386 ir 189247±9134 imp/min. Tuo tarpu lipopolisacharidu stimuliuotų sergančiųjų periodontitu periferinio kraujo neutrofilinių leukocitų nuo luminolo priklausoma chemiliuminescencija buvo net penkis kartus didesnė už analogišką asmenų, kurių priedančio audiniai sveiki, ir sudarė atitinkamai – 13261±1251 ir 2627±638 imp/min.

Išvados. Tyrimų duomenys rodo sudėtingą sergančiųjų periodontitu periferinio kraujo neutrofilinių leukocitų okcidacinės funkcijos priklausomumą nuo stimuliatoriaus prigimties, todėl tikslinga ir toliau tyrinėti šių ląstelių oksidacinę funkciją, įvertinti jos įtaką priedančio audinių uždegiminės kilmės ligoms pasireikšti.

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