Peripheral Blood Leukocytes Interleukin-1 Beta (IL-1β) Cytokine Hyper-Reactivity in Chronic Periodontitis

Authors' Contribution:
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Data Collection B
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Background: Levels of pro-inflammatory cytokine (IL-1β) released by peripheral blood leukocyte medium (PBLM), isolated from chronic periodontitis patients (P) before therapy and matched to controls, were determined in the presence or absence of non-opsonized *Escherichia coli* and *Staphylococcus aureus*.

Material/Methods: In this investigation, 26 patients with untreated, severe, generalized, chronic periodontitis and 26 healthy subjects (H) were enrolled. Periodontal status was assessed by measuring bleeding on probing (BOP), clinical attachment loss (CAL), probing pocket depth (PPD), and Ramfjord index (PDI). The levels of IL-1β (μg/ml) were assayed by a standard Immunoenzymetric Assay Diasource IL-1β ELISA kit in PBLM.

Results: Our study showed that the values of IL-1β levels in PBLM of the P group (stimulated with non-opsonized *E. coli* and *S. aureus*) were significantly higher than in the analogous medium of H group subjects ($P<0.001$). All correlations between the cytokine levels of IL-1β in the samples of PBLM (stimulated with non-opsonized *E. coli* and *S. aureus*) and clinical parameters such as BOP, PPD, CAL, and PDI were significantly higher in the group of patients with periodontitis.

Conclusions: Levels of IL-1β secreted by leukocytes may help measure severe, generalized, chronic periodontitis, and can be predictive of future detrimental clinical sequelae associated with chronic periodontitis.

MeSH Keywords: Chronic Periodontitis • Leukocytes, Mononuclear • Receptors, Cytokine

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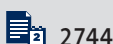
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Background

Periodontitis is both a polymicrobial and multifactorial disease in which clinical measures such as periodontal pocket depth, clinical attachment level, BOP, and resorption of alveolar bone provides a retrospective history of disease and current status, but has limited predictive value. Cross-sectional data analysis identified putative biomarkers from anaerobic pathogens and saliva that were strongly related to periodontal disease status [1]. The host response due to persistent bacterial challenge disrupts homeostatic mechanisms and results in the recruitment of polymorphonuclear neutrophils, monocytes/macrophages, and the subsequent release of mediators, including pro-inflammatory cytokines, matrix metalloproteinases, arachidonic acid metabolites, and reactive oxygen species (ROS), as well as release of mediators for osteoclastic bone resorption and connective tissue destruction, which are thought to play crucial roles in the pathogenesis of periodontal disease [2,3].

Members of the IL-1 family are thought to be the key mediators of host response to microbial invasion, inflammation, tissue injury, and immunological reactions. Both directly and indirectly, IL-1 plays a role in the activation of T cells and hematopoietic stem cells, the induction of fever and acute-phase proteins, the degradation of cartilage, and the healing of wounds [4]. IL-1β is mainly expressed by macrophages and dendritic cells, but gingival fibroblasts, periodontal ligament cells, and osteoblasts can also secrete it [5].

Interleukin (IL) -1β is an important mediator of inflammatory response and is involved in cell proliferation, differentiation, and apoptosis, as well as in the pathophysiology of periodontitis [6].

The majority of studies investigating cytokines levels in chronic periodontitis (CP) patients have been performed on plasma, gingival crevicular fluid, tissue and biopsy samples, and have shown higher levels in disease. Some authors demonstrated no significant difference in neutrophil IL-8, IL-1β, or TNF-α release in CP as compared with healthy subjects in the absence or presence of granulocyte macrophage colony-stimulating factor or opsonized *Staphylococcus aureus* [7]. Several studies have reported that gingival crevicular fluid (GCF) volume and IL-1β level in samples reflect periodontal disease severity and that these variables are better than PPD and BOP as markers for gingival inflammation [8]. Recent reports have demonstrated that salivary and biofilm biomarkers have potential for the identification of periodontal disease progression or stability [9]. Numerous mediators of chronic inflammation and tissue destruction have been detected in whole saliva of patients with periodontitis [10].

Despite the serum levels of various cytokines in periodontitis patients investigated in multiple studies, the results remain incomplete and controversial [11]. The specific mechanisms involved in neutrophil cytokine release are incompletely defined and it is not clear for how long neutrophils store cytokines and the time required for the synthesis, activation, and release of pro-cytokine molecules following stimulation [12].

Therefore, we hypothesized that non-opsonized bacteria *E. coli* and *S. aureus* can modulate the release of IL-1β from leukocytes and convert it to a pattern that is more destructive of the tooth-supporting tissues. To test this hypothesis, the effect of non-opsonized *E. coli* and *S. aureus* on the production of this cytokine IL-1β in the PBLM of patients with CP was investigated, and the associations between clinical and biochemical parameters were analyzed.

Material and Methods

Sampling

Fifty-two nonsmoking subjects (men and women aged 30–50 years) were included in this study. They received dental care at the Department of Odontology, Medical Academy of the Lithuanian University of Health Sciences. All experiments were conducted in accordance with the rules and regulations approved by the Regional Bioethics Committee, City of Kaunas (No. BE-2-76) and in accordance with the Declaration of Helsinki. All volunteers involved in this study had to read and sign the informed consent form approved by the Regional Bioethics Committee, City of Kaunas.

Patient diagnosis with untreated, generalized, chronic periodontitis was confirmed by clinical examination and X-ray analysis based on the criteria proposed by the World Workshop for Classification of Periodontal Diseases and Conditions in 1999 [13]. All participants were assigned to 1 of 2 groups: 26 patients with untreated, severe, generalized, chronic periodontitis (P), and 26 healthy subjects (H). Periodontal status was assessed by measuring bleeding on probing (BOP), CAL, PPD, and Ramfjord index (PDI) [14]. BOP was recorded as present or absent within 30 s after probing with the periodontal probe. pocket probing depth was measured in all patients at 6 sites per tooth with a periodontal probe (PCP 15, Stoma Storz am Mark, Germany) and CAL was obtained by measuring only interproximal sites.

Clinical diagnosis of untreated generalized CP was defined as follows: ≥30% of periodontal sites with PPD ≥4 mm; ≥20% of periodontal sites with interproximal CAL of >2 mm; and ≥30% of periodontal sites showing BOP and PDI. In patients with CP, X-ray images were made for diagnostics and the treatment plan, along with radiographic bone loss ≥30% of the root length (non-first molar or incisor sites), and healthy subjects

had ready X-ray images. Periodontally healthy patients at the time of the oral examination had no sites with PPD >3 mm, no CAL >2 mm, no radiographic evidence of bone loss, no BOP, and PDI was evaluated. Systemic exclusion criteria included the presence of cardiovascular and respiratory diseases, systemic inflammatory conditions such as diabetes mellitus, non-plaque-induced oral inflammatory conditions, immunodeficiency, and current pregnancy or lactation. All participants were non-smokers, had no anti-allergy medications, antibiotics, or antioxidants used over the 6-month period prior to the study. All subjects were screened for suitability, and were classified according to the degree of BOP, PPD, CAL, and PDI, and age.

Reagents

Hank's balanced salt solution was obtained from Sigma Chemical Co. (St. Louis, MO). Plastic vials and other disposable plastic utensils were obtained from Carl Rot GmbH & Co KG (Karlsruhe, Germany).

Leukocytes isolation and culture

Peripheral blood was obtained between 9:00 and 10:00 AM from the patients, and was matched to that of healthy control volunteers within 30 min. Fifteen milliliters of blood was collected from the antecubital vein into Vacutainers containing heparin (20 IU/ml) as the anticoagulant. Samples were centrifuged with a force of 500 RCF for 35 min at 20–25°C.

Then, the supernatant plasma rich in leukocytes was aspirated. The leukocyte count in the plasma was levelled to 1×10^9 cells/l with the help of Hank's balanced salt solution. Cells were counted using a Sysmex xe-5000 hematological blood analyzer (Sysmex Corporation, Japan). Unstimulated and stimulated leukocytes were used in the study. The samples of PBLM were prepared using the Timm et al. method [15]. The stimulation of the cells was by soluble inductors. Myelin basic protein had a direct stabilizing action on the membrane of lysosomes and induced an increased secretory reaction of cells after irritation. *E. coli* (*Ec*; ATCC 25922) and *S. aureus* (*Sa*; 25923) were grown in the Laboratory of Microbiology at the Medical Academy of the Lithuanian University of Health Sciences. Samples of the *E. coli* and *S. aureus* culture for the investigations were used within 24 h at a concentration of 3×10^6 cells/ml.

Experimental protocol

The experiments were performed with unstimulated and stimulated leukocytes from the periodontally healthy subjects and patients with periodontitis. The testing system was prepared as follows: 0.475 ml of PBLM was taken for each test tube. Then, 0.025 ml of phosphate buffer was added to the first sample (unstimulated control); 0.025 ml of non-opsonized *E. coli*

(IL-1β *E. coli*) was added to the second sample; and 0.025 ml of non-opsonized *S. aureus* (IL-1β *S. aureus*) was added to the third sample. The prepared test samples were placed into an incubator at 37°C and kept for 5 h. Then, the levels of IL-1β were measured in the medium.

Determination of leukocytes cytokine release

The level of IL-1β was assayed by standard immunoenzymetric assay using Diasource IL-1β ELISA kits (Diasource ImmunoAssays S.A., Belgium). All assay procedures were performed according to the manufacturer´s recommendations. The level of IL-1β µg/ml was assessed. The experiments with leukocytes for each patient were performed in triplicate.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY). Descriptive statistics included mean, median, standard deviation, and minimum and maximum values. Since most variables were not normally distributed, the Mann-Whitney U was used to compare P to H subjects, the Wilcoxon signed rank test was used to compare unstimulated to stimulated values within the same subjects, and Spearman correlation was used to assess the correlation between IL-1β levels and clinical parameters. The level of statistical significance was 0.05.

Results

The descriptive statistics of the clinical parameters for individuals in the P and H groups are presented at the top portion of Table 1. Groups of patients did not differ by age, with an average age ~45 years. BOP was present in group P but not in group H, with a statistically significant difference (*P*<0.001). PPD in group P was significantly deeper than that in group H (*P*<0.001), and the CAL and PDI values in group P were significantly higher than in group H (*P*<0.001).

The data in Table 2 show that the leukocyte count in the PBLM of the groups studied did not differ significantly (*P*>0.1). The results of the levels of IL-1β in the PBLM assays for P and H groups of patients are presented at the bottom of Table 3. There was no difference between P and H patients based on the level of unstimulated IL-1β (*P*= 0.805), but group P patients showed higher levels than group H patients in IL-1β when stimulated by *E. coli* (*P*<0.001) and *S. aureus* (*P*=0.032). In group H patients there was no difference in the IL-1β levels between unstimulated and *E. coli*-stimulated (*P*=0.416) or between unstimulated and *S. aureus*-stimulated (*P*= 0.97) cases (Table 4). In contrast to H group patients, patients from group P showed an increase in IL-1β levels when stimulated by *E. coli* (*P*<0.001) and stimulated

Table 1. Clinical parameters (means and standard deviations) of patients with chronic periodontitis and healthy subjects.

Clinical parameters	P group (n=26)	H group (n=26)	P
BOP	0.78±0.12	0	<0.001
CAL	4.70±0.26	0.73±0.34	<0.001
PPD	4.50±0.43	0.60±0.01	<0.001
PDI	5.01±0.28	0.18±0.08	<0.001
Age	44.3±7	45.1±7	0.814

H – healthy subjects; P – patients with untreated severe generalized chronic periodontitis; BOP – the bleeding on probing; CAL – the clinical attachment loss; PPD – pocket probing depth; PDI – Ramfjord index.

Table 2. Leukocyte count and percentage distribution in peripheral blood incubation medium.

Groups	Leukocyte count (1×10⁹/l)	Leukocytes in incubation medium		
		Differential leukocytes count		
		Granulocytes %	Lymphocytes %	Monocytes %
H group (n=26)	10.2±0.11	43.7±6.5	54.4±7.5	2.7±1.0
P group (n=26)	10.4±0.14	45.4±7.5	52.8±8.2	2.7±1.4
P	P>0.1	P>0.1	P>0.1	P>0.1

H – healthy subjects; P – patients with untreated severe generalized chronic periodontitis.

Table 3. The levels of interleukin IL-1β (means and standard deviations) produced by the leukocytes (unstimulated and stimulated by *E. coli* and *S. aureus*) of healthy subjects and patients with chronic periodontitis.

Biochemical parameters	P group (n=26)	H group (n=26)	P
IL-1β unstimulated	0.0180±0.0049	0.0178±0.0042	0.805
IL-1β stimulated *E. coli*	0.0289±0.0031	0.0180±0.0006	<0.001
IL-1β stimulated *S. aureus*	0.0247±0.0129	0.0180±0.0087	0.032

H – healthy subjects; P – patients with untreated severe generalized chronic periodontitis.

Table 4. Significance of within group differences of biochemical parameters.

Biochemical parameters	P group (n=26)	H group (n=26)
IL-1β unstimulated *vs.* IL-1β stimulated *E. coli*	<0.001	0.416
IL-1β unstimulated *vs.* IL-1β stimulated *S. aureus*	0.015	0.970
IL-1β stimulated E. coli *vs.* IL-1β stimulated *S. aureus*	0.078	0.684

H – healthy subjects; P – patients with untreated severe generalized chronic periodontitis.

by *S. aureus* (P=0.015) as compared to unstimulated. Table 5 represents the results of correlation analysis. The levels of unstimulated IL-1β did not correlate with any clinical parameters. *S. aureus*-stimulated IL-1β levels showed low to moderate correlations, with significant correlations of IL-1β levels with

PPD (P=0.02), PDI (P=0.03), and CAL (P=0.02). *E. coli*-stimulated IL-1β levels were strongly and significantly correlated with BOP (P<0.001), PPD (P<0.001), PDI (P<0.001), and CAL (P<0.001).

No age correlation was found with any of the IL-1β levels.

Table 5. Spearman correlations for clinical parameters and cytokine IL-1β levels in the peripheral blood leukocyte medium stimulated by *E. coli* and *S. aureus* and unstimulated.

Parameters	BOP	PPD	PDI	CAL	Age
IL-1β unstimulated	−0.09	−0.14	0.01	0.01	0.00
IL-1β stimulated with *S. aureus*	0.24	0.33*	0.30*	0.33*	−0.20
IL-1β stimulated with *E. coli*	0.83**	0.76**	0.73**	0.75**	−0.05

Correlations are significant at ($P<0.05$) *, ($P<0.001$) **. BOP – the bleeding on probing; PPD – the pocket probing depth; PDI – Ramfjord index; CAL – the clinical attachment loss.

Discussion

Our data from this study demonstrate that peripheral blood leukocytes obtained from patients with chronic periodontitis and stimulated with *E. coli* and *S. aureus* release greater amounts of cytokine IL-1β as compared with those from healthy subjects. The leukocyte count in the PBLM did not have any substantial influence on the results of the groups studied.

They may exhibit a hyper-reactive phenotype in terms of cytokine production in response to a variety of stimuli, including the periodontitis-associated periodontopathogens. The dysregulated neutrophil phenotype in chronic periodontitis pathogenesis is significant because cytokines regulate many different functions of the inflammatory immune response known to be altered in periodontitis, including neutrophil reactive oxygen species generation, extracellular trap release, and directional chemotactic accuracy [16,17].

Although specific bacteria are essential for the initiation and progression of periodontal disease, the tissue destruction caused by periodontitis is the result of the imbalance between the protective and destructive host mechanisms that are initiated by an infection [18].

These interactions lead to the secretion of various cytokines by host cells. The innate host response initially involves the recognition of microbial components as "danger signals" by host cells and the subsequent production of inflammatory mediators. Toll-like receptors (TLRs) are expressed by resident cells and leukocytes in the periodontal environment, and they activate the innate immune response by binding to various bacterial components (e.g., lipopolysaccharide, bacterial DNA, diacyl lipopeptides, and peptidoglycan) [19]. Recent studies described a role of both TLR-2 and TLR-4 in the recognition of periopathogens such as *A. actinomycetemcomitans, P. gingivalis,* or *T. forsythensis* [20]. After TLR activation, an intracellular signaling cascade is stimulated, leading to the activation of transcription factors and to subsequent inflammatory cytokine expression, antibody and leukocyte migration, and osteoclastogenesis [21]. Bodet et al. reported a polymicrobial challenge involving *P. gingivalis, T. denticola,* and *T. forsythia,*

which resulted in an increased pro-inflammatory cytokine production in an *ex vivo* whole-blood model [22]. Recent results have suggested that infection with *Peptostreptococcus micros, Fusobacterium periodontium, Eubacterium nodatum, Eikenella corrodens,* and *Campylobacter rectus* may be an important indicator of inflammatory periodontal disease development [23].

In the present study, *E. coli* and *S. aureus* bacteria were chosen as representatives of Gram-negative and Gram-positive bacteria displaying coming periodontal antigens. *E. coli* is not a typical periodontopathogen, but there is classic lipopolysaccharide (LPS) in the wall of *E. coli*. LPS is a common component in all Gram-negative bacteria, which are the most common periodontopathogens. As an outer-membrane component of Gram-negative bacteria, LPS is a potent activator of monocytes and macrophages, and it triggers the abundant secretion of many cytokines from macrophages, including IL-1, IL-6, and TNF-α [24]. *S. aureus* is a Gram-positive bacterium that has peptidoglycan as the main component in the cell wall. Peptidoglycan is also a common component of the cell wall in periopathogens. Peptidoglycan recognition in the protein-peptidoglycan complexes increases monocyte/macrophage activation and enhances the inflammatory response [25].

IL-1β and TNF-α are critical determinants of the progression of periodontitis. These cytokines can induce the expression of adhesion molecules and secondary mediators that facilitate and amplify the inflammatory response, matrix metalloproteinase production, and bone resorption [2]. Various studies have shown that IL-1β stimulates the plasminogen activator (PA) system and inhibits the PA inhibitor (PAI) system [26]. Similarly, Oh et al. [8] assessed the effects of initial periodontal therapy (tooth brushing instruction, scaling, and root planning) on clinical periodontal parameters (BOP and PPD) and IL-1β levels in gingival crevicular fluid from chronic periodontitis patients. Significant improvements in PPD and BOP were observed at 2 and 4 months after periodontal initial therapy in deep PPD sites only. In contrast, gingival crevicular fluid volume and IL-1β level were lower at 2 and 4 months after initial therapy at all sites. These results suggest that gingival crevicular fluid volume and IL-1β levels in samples reflect disease severity and that those variables are better than PPD and BOP as markers for gingival inflammation.

Suzuki et al. found no significant difference between the chronic periodontitis and aggressive periodontitis groups regarding gingival crevicular fluid levels of IL-1 [27]. Findings published by Toyman et al. suggest that IL-1β plays an important role in tissue destruction by stimulating matrix metalloproteinase-3 (MMP-3) and tissue-type PA (t-PA) in the etiopathogenesis of periodontal disease, and the evaluation and comparison of gingival crevicular fluid IL-1β, MMP-3, t-PA, and PAI-2 levels may be used as markers to assess the progression of periodontal diseases [28]. These proteins are biochemically active in processes integrally involved in inflammation, connective tissue degradation, and osteoclast-modulated alveolar bone turnover [10]. The small number of macrophages, mononuclear cells, and neutrophils in gingival tissues can be found in healthy subjects. These cells, including resident fibroblasts and endothelial cells, can all synthesize and release IL-1β. Thus, genetic factors, local microbial quality, individual immunologic response, and activity of different combinations of inflammatory mediators may influence IL-1β levels [29].

Our study revealed significant correlations between biochemical parameters in the samples of PBLM, the cytokine levels of IL-1β (stimulated with non-opsonized *E. coli, S. aureus),* and clinical parameters (BOP, PPD, PDI, CAL and PPD, PDI, and CAL) in group of patients with CP.

Previous studies have demonstrated that CP is characterized by neutrophils that constitutively exhibit cytokine hyper-reactivity, the effects of which could modulate local and systemic inflammatory-immune responses and influence the risk and severity of periodontitis-associated systemic inflammatory

diseases [30]. These findings need to be confirmed in larger studies with different diagnostic sensitivity and treatment of CP.

Our study introduces the idea that greater amounts of released cytokine IL-1β may be predictive of future detrimental clinical sequelae associated with chronic periodontitis.

Conclusions

The levels of IL-1β secreted by leukocytes may help measure severe generalized chronic periodontitis, and can be predictive of future detrimental clinical sequelae associated with chronic periodontitis. We tested our hypothesis that *E. coli* and *S. aureus* can be used as activators of IL-1β secretion. *E. coli* and *S. aureus* are LPS and peptidoglycan sources and are common to periodontopathogens. We found that they clearly activate leukocytes to produce IL-1β in periodontitis patients.

Limitations of study

The small numbers of individuals in both subgroups may have affected our findings.

A larger study is necessary to explore the significance of cytokine IL-1β in the progression of chronic periodontitis.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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