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Effects of ß-1.3/1.6 glucan on cytokines production by leukocytes in vitro

Research Article

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Abstract: Background. The aim of the study was to investigate the immunomodulating effect of ß-1,3/1,6 glucan from yeast on the production of interleukin-4 (IL-4), interleukin-5 (IL-5) in a medium of peripheral venous blood leukocytes from patients with periodontitis. Material/Methods. The study was performed using venous blood of 22 patients, aged 20 to 45 years, suffering from untreated severe chronic generalized periodontitis confirmed by clinical and radiological examination, and 22 periodontally healthy subjects. The effects of ß-1,3/1,6 glucan on the production of the cytokines IL-4 (pg/ml) and IL-5 (pg/ml) levels by unstimulated and stimulated leukocyte incubation medium with unopsonized *E. coli* were determined using the Enzymes Linked Immunosorbent Assay (ELISA) method. Results. We found that the incubation medium of stimulated leukocytes by unopsonized *E. coli* and treated with 0.2 mg and 0.4 mg of ß -1,3/1,6-glucan of patients with periodontitis produced significantly higher ($P<0,001$; $P<0,001$) levels of IL-4 and IL-5 than the analogous medium of healthy subjects. Conclusion. This study indicates that ß-1,3/1,6-glucan may significantly increase production of IL-4 and IL-5 cytokine levels in a stimulated peripheral blood leukocytes incubation medium from periodontitis patients.

Keywords: *Periodontal disease • ß-1,3/1,6 glucan • Interleukin-4 • Interleukin-5 • Predictors • Markers • Microalbuminuria*

1. Background

The most common form of periodontitis is a variably progressive, dynamic pathologic process that causes attachment loss, destroys the alveolar bone supporting a tooth, and terminates with tooth loss [1]. Periodontal inflammation is associated with the formation of an infiltrate consisting of polymorphonuclear leukocytes, monocytes, and lymphocytes [2]. Not only do these neutrophils migrate efficiently, but also appear to be hyperactive, primed to release enhanced levels of bactericidal molecules such as oxygen radicals, inflammatory mediators such as inflammatory cytokines and matrix-degrading enzymes, and osteoclast activation; these factors are considered responsible for the tissue destruction. Leading authors of these studies have concluded that primed or hyperactive neutrophils are the perpetrators of periodontitis, rather than the protectors [2,3,4].

The regulation of cytokine levels plays an important role in the course of the inflammatory processes

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in the periodontal tissues. Different T-cell subsets are associated with various cytokine secretion profiles that regulate different aspects of immune response and contribute to upregulated cytokine activity. Th1 cells produce mainly IL-1β, IL-2, IL-12, interferon (IFN)-y, and tumor necrosis factor (TNF)-a, which together induce the cellular immune response. Th2 cells produce mainly IL-4, IL-5, IL-6, IL-10, IL-13, which induce the humoral immune response [3,5]. Patients suffering from chronic periodontitis have been shown to express elevated levels of pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, in gingival tissues and gingival crevicular fluid [6,7]. Other studies have suggested that a Th1 inflammatory immune response is associated with chronic periodontitis progression, whereas an anti-inflammatory Th2 immune response is not associated with progression of periodontal disease [8,9]. Protective aspects of the host immune response include recruitment of neutrophils, production of protective antibodies, and possibly, the release of anti-inflammatory cytokines [4].

ß –glucans belong to a group of natural products that are physiologically active compounds useful in treating and/or preventing various diseases. These polysaccharides, non-cellulosic polymers of ß-glucose, have glycosidic bonds in position ß (1→3) and have a portion of ß (1→6) bound glucose molecules. They are isolated from different fungi, but are also present in other sources such as cereal bacteria and seaweed [10]. The best known effects of ß-glucan consist of the augmentation of phagocytosis of professional phagocytes – granulocytes, monocytes, macrophages and dendritic cells.

Macrophages, considered to be the basic effector cells in host defense against bacteria, viruses, parasites and tumor cells, play the most important role [10,11].

The aim of the present research was to analyse *in vitro* the influence of ß-1,3/1,6 glucan on the levels of anti-inflammatory cytokines (IL-4, IL-5) in a medium of peripheral venous blood leucocytes from patients with periodontitis.

2. Material and methods

2.1. Subjects

The study was performed in a sample of 44 (22 females and 22 males) persons treated in the Department of Odontology at Medical Academy of Lithuanian University of Health Sciences. The age of the examined patients ranged from 20 to 45 years; they suffered from untreated severe generalized chronic periodontitis as confirmed by clinical examination and X-ray analysis.

Table 1. Analysis scheme

Peripheral venous blood unstimulated leukocytes or stimulated with unopsonized *E.coli* leukocytes medium	Control sample 0.475 ml	ß-glucan 0.2 mg sample 0.475 ml	ß-glucan 0.4 mg sample 0.475 ml
Phosphate buffer	0.025 ml		
ß-glucan solution		0.025 ml	
ß-glucan solution			0.025 ml

The extent of periodontal destruction was determined according to the Russell [12] periodontal index (PI). Patients with very marked signs of periodontitis (PI >6.0 points) were included in this study. The control group consisted of 22 periodontally healthy subjects: no pathological dentogingival pockets, no bleeding on probing, (PI<0.02 points) (Table 1). None of those examined reported concomitant disorders of the haemopoietic system, autoimmune disease, chronic viral or bacterial infection, or any other systemic disorders. Patients had not used medications and antioxidant drugs in the 6-month period prior to the study. All participants were non-smokers and none of the women were pregnant.

All experiments were conducted in accordance with the rules and regulations approved by the Kaunas Regional Bioethics Committee (approval Nr.BE-2-76). All subjects involved in this study signed the form of consent approved by the Kaunas Regional Bioethics Committee.

2.2. Reagents

Hank's balanced salts solution were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Plastic vials and other disposable pieces of plastic were obtained from Carl Rot GmbH & Co KG (Karlsruhe, Germany). ß-1,3/1,6 glucan from yeast was obtained from the Gee Lawson company (http://www.geelawson.co.uk/). *Escherichia coli* ATCC 25922 (*E. coli*) strains were grown in the Laboratory of Microbiology at Medical Academy of Lithuanian University of Health Sciences. Specimens of *E. coli* culture for the investigations were used within 24 hours of growth at a concentration $3x1^6$cells/ml.

2.3. Blood sampling and leukocytes isolation

Fifteen milliliters of peripheral venous blood were taken from patients with periodontitis, who had abstained from morning meals, using a sterile vacuum test-tube containing heparin (20 IU/ml). Subsequently, the supernatant containing leukocytes was aspirated and the leukocyte count was standardized to 1 x 10^9/l cells using Hank's balanced salt solution. The samples of leukocyte

incubation medium was prepared by the Timm et al. method [13]. Cells were counted using a hematological blood analyzer ADVIA 2120 (Siemens Healthcare Diagnostics, Dublin, Ireland).

2.4. ß- glucan

(1→3),(1→6)-β-glucan from yeast (Gee Lawson) was dissolved in phosphate buffer. To each test sample, 0.025 ml of (1→3), (1→6)-β-glucan solution in 8 mg/ml or 16 mg/ml concentration was added, yielding a final ß-glucan dose of 0.2 mg or 0.4 mg per sample, respectively.

2.5. Experimental protocol

Two systems of peripheral venous blood leukocytes medium were prepared. The first system was prepared as follows: 0.475 ml of venous blood leukocytes was added to every test tube (1×10^9/l unstimulated leukocytes). Then, 0.025 ml of phosphate buffer was added to the first sample (control medium); to the second sample, 0.025 ml of ß-1,3/1,6 glucan solution in the amount of 0.2 mg per test tube, was added; to the third, 0.025 ml containing 0.4 mg of ß-1,3/1,6 glucan.

The second system was prepared as follows: 1.425 ml (1×10^9/l leukocytes) human blood leukocytes were incubated with unopsonized *E. coli* (3×10^6 cells/ml) at 37°C for 45 min, and 0.475 ml of venous blood with stimulated leukocytes was taken for every test tube. Then 0.025 ml of phosphate buffer was added to the fourth sample (control medium); to the fifth, 0.025 ml containing 0.2 mg of β-1,3/1,6 glucan per test tube; to the sixth, 0.025 ml containing 0.4 mg of β-1,3/1,6 glucan.

Both systems were then placed into a thermostat and maintained for 5 hours at 37°C. The IL-4 and IL-5 levels were then measured.

We chose 5 hours after considering the results presented in other studies [14,15,16]. In [14] the monocytes were stimulated by b-glucan laminarin and incubated for 6 hours. In another study [15], the effect of seven b-glucans that differed in origin and structure were tested on peripheral blood mononuclear cells and neutrophils; the stimulation time was 24 hours. In the third study [16], human peripheral blood mononuclear cells were treated with b-D-glucan for 4 and 18 hours. Thus, if b-glucan as an active ingredient can have an effect on the different cells mentioned above from these three studies, [14,15,16], we can make the decision for the incubation time to be 5 hours.

The experiments with leukocytes of each patient, unstimulated and stimulated by unopsonised *E. coli*, were performed in triplicates.

2.6. Cytokine determination

In the samples of peripheral venous blood leukocytes, the cytokines levels of interleukin-4 (IL-4) and interleukin-5 (IL-5) were determined by the Enzymes Linked Immunosorbent Assay (ELISA) method. Human interleukin-4, interleukin-5 ELISA Kit (Anogen, Canada) was used for the *in vitro* quantitative determination of human IL-4 and IL-5 levels in the peripheral venous blood leukocyte medium.

2.7. Statistical analysis

Descriptive statistics were used to summarize data using the mean and standard deviation. Differences of indices distribution between studied groups were tested using the nonparametric Mann-Whitney test. Differences of indices distribution within groups were tested using nonparametric Friedman and Wilcoxon's signed-ranks tests. The significance level was set at 0.05. Data statistical analysis was performed using the statistical analysis package SPSS 15.

3. Results

The measurement of IL-4 and IL-5 cytokine production was performed in a leukocyte medium produced from venous blood of patients with untreated chronic generalized periodontitis and in subjects with intact periodontal tissues after the immunomodulating effect of ß-glucan in different concentrations; the analysis scheme is given in Table 1. The means and standard deviations of the clinical indices of studied groups are summarized in Table 2. The mean age of the subjects in the periodontitis and healthy groups (32.1 ± 1.2 years and 32.0 ± 1.6 years, respectively) did not differ significantly ($P>0.05$). The mean PI index of the patients with periodontitis was 6.5 ± 0.21, significantly higher than that of the subjects with intact periodontium, at 0.02 ± 0.01 ($P<0.001$).

It is evident from the data presented in Table 3 that there were no statistically significant differences in the peripheral blood of neutrophils and eosinophils (P>0.05; P>0.05) in the group of periodontitis patients

Table 2. Descriptive clinical indices in study groups

Studied groups	Age (years)		PI index (points)	
	mean	SD	mean	SD
Healthy subject group n=22	32.0	1.6	0.02*	0.01
Periodontitis patient group n=22	32.1	1.2	6.5*	0.21

PI – Russell periodontal index (points)
**P <0.001*

Table 3. Laboratory testing data, peripheral blood.

Studied groups	Healthy subject group n=22		Periodontitis patient group n=22		P
	%	Count (x 10^9/l)	%	Count (x 10^9/ l)	
Neutrophils	56.6 ± 3.36	3.8 ± 0.1	62.7 ± 2.7	3.9 ± 0.3	>0.05
Lymphocytes	27.51 ± 0.87	1.63 ± 0.09	34.3 ± 0.61	2.3 ± 0.1	<0.001
Eosinophils	2.52 ± 0.10	0.15 ± 0.01	2.3 ± 0.18	0.14 ± 0.02	>0.05

Table 4. Leukocyte count and percentage distribution in incubation medium

Groups	Leukocytes in incubation medium			
	Leukocyte count (1x10^9/l)	Differential leukocyte count		
		Granulocytes %	Lymphocytes %	Monocytes %
Healthy subject group	10.0±0.12	42.8±6.6	54.5±7.6	2.7±1.1
Periodontitis patient group	10.0±0.15	46.5±7.6	50.9±8.3	2.6±1.5
P		P>0.1	P>0.1	P>0.1

Table 5. IL-5 and IL-4 levels in unstimulated leukocytes medium and treated with ß -1,3/1,6-glucan.

Index	Treatment	Healthy subject group n=22		Periodontitis patient group n=22		P
		mean	SD	mean	SD	
IL-5 in unstimulated leukocytes medium +	0.4 mg BG	0.0371	0.0028	0.0391	0.0042	>0.05
	0.2 mg BG	0.0375	0.0027	0.0390	0.0045	>0.05
	Control	0.0394	0.0033	0.0400	0.0049	>0.05
IL-4 in unstimulated leukocytes medium +	0.4 mg BG	0.0376	0.0081	0.0500	0.0214	<0.05
	0.2 mg BG	0.0392	0.0081	0.0470	0.0020	>0.05
	Control	0.0370	0.0019	0.0350	0.0036	>0.05

BG–ß-1,3/1,6 glucan 0.2 mg and 0.4 mg; IL-5, interleukin -5; IL-4, interleukin-4 (pg/ml).

Table 6. IL-5 and IL-4 levels in leukocyte medium stimulated by unopsonized E. coli and treated with ß -1,3/1,6-glucan.

Index	Treatment	Healthy subject group n=22		Periodontitis patient group n=22		P
		mean	SD	mean	SD	
IL-5 in stimulated leukocytes medium +	0.4 mg BG	0.0380	0.0012	0.0830	0.0013	<0.001
	0.2 mg BG	0.0400	0.0071	0.0650	0.0044	<0.001
	Control	0.0400	0.0090	0.0410	0.0012	>0.05
IL-4 in stimulated leukocytes medium +	0.4 mg BG	0.0411	0.0059	0.0790	0.0044	<0.001
	0.2 mg BG	0.0414	0.0108	0.0600	0.0098	<0.001
	Control	0.0390	0.0011	0.0400	0.0049	>0.05

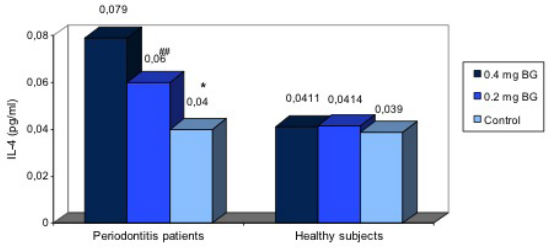
BG, ß-1,3/1,6 glucan 0.2 mg and 0.4 mg; IL-5, interleukin -5; IL-4, interleukin-4 (pg/ml) leukocytes stimulated by unopsonized E. coli.

as compared with the healthy subjects, whereas the difference between lymphocyte concentrations was statistically significant (P<0.001). The data in Table 4 show that the leukocyte count in the incubation medium of the groups studied did not differ significantly (P >0.1).

The results for the analyzed variables of IL-4 and IL-5 levels in two systems of venous blood leukocytes assays of periodontitis patients and healthy subjects following the effects of ß –glucan with different concentrations are given in Tables 5 and 6 and Figures 1, 2.

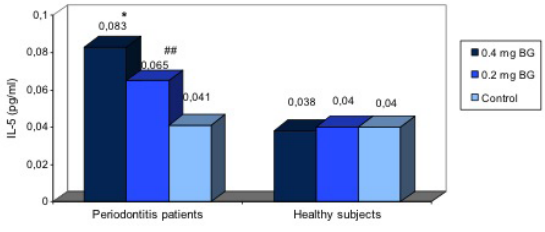
Our findings showed no significant differences in the IL-4 levels in unstimulated control leukocyte medium and the leukocyte medium treated with 0.2 mg of ß-1,3/1,6-glucan when comparing patients with periodontitis to healthy subjects (P>0.05; P>0.05). Only treatment with 0.4 mg of ß-1,3/1,6-glucan induced a significant increase in the IL-4 level in the unstimulated leukocyte medium of patients with periodontitis as compared with that in healthy subjects (P<0.05). Interestingly, no significant differences in the IL-5 levels in the unstimulated control

Figure 1. Comparison of IL-4 levels in stimulated leukocyte medium within periodontitis patient and healthy subject groups.



* p<0.001 compared to 0.4 mg BG and 0.2 mg BG
p<0.001 compared to 0.4 mg BG

Figure 2. Comparison of IL-5 levels in stimulated leukocyte medium in periodontitis patient and healthy subject groups



* p<0.01 compared to 0.2 mg BG and control
##p<0.01 compared to control

leukocyte medium and leukocyte medium treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan were documented in patients with periodontitis as compared with healthy subjects (P>0.05; P>0.05; P>0.05). After the stimulation of the leukocyte medium with unopsonized *E. coli*, significant differences in the IL-4 and IL-5 levels were observed between patients with periodontitis and healthy subjects. Patients with periodontitis showed significantly higher (P<0.001; P<0.001) IL-4 and IL-5 levels in the leukocyte medium stimulated with unopsonized E. coli and treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan than did the analogous leukocyte medium of healthy subjects.

Intragroup comparison of the IL-4 levels in patients with periodontitis showed significant increases between the leukocyte medium stimulated with unopsonized *E. coli* as well as that treated with 0.2 or 0.4 mg of ß-1,3/1,6-glucan and the control leukocyte medium (P<0.001; P<0.001). Intragroup comparison of the IL-4 levels in healthy subjects showed no significant differences between the leukocyte medium stimulated with unopsonized E. coli as or those treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan and control leukocytes medium (P>0.05; P>0.05). Patients with periodontitis no showed significantly differences in IL-5 levels in the unstimulated leukocyte medium treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan than in the control leukocytes medium (P>0.05;P>0.05); however, IL-4 levels in the unstimulated leukocyte medium treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan were significant higher (P<0.01; P<0.01) than that of the control leukocyte medium. Interestingly, the patients with periodontitis showed significantly higher IL-5 levels when comparing the leukocyte medium stimulated by unopsonized *E. coli* or that treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan and the control leukocyte medium (P<0.01; P<0.01, respectively). However, in healthy subjects, the IL-5 levels were not significantly different when comparing the leukocyte medium stimulated by unopsonized E. coli or that treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan and the control leukocyte medium (P>0.05; P>0.05).

4. Discussion

Innate immune cells have been shown to play an important role in periodontal bone resorption [17]. Released granule components from infiltrating leucocytes, such as lysosomal enzymes and reactive oxygen species normally intended to degrade ingested microbes, can also lead to tissue destruction and amplification of the inflammatory response [18]. It has been suggested that hyper-responsiveness of monocytes to the products of dental plaque, especially the endotoxin of gram-negative bacteria, and also the secretion of high levels of pro-inflammatory cytokines, may have a role in the pathogenesis of periodontal disease [19].

The data obtained by Andrukhov et al. [20] showed that an increased content of some bacteria (*Tannerella forsythia, Trepomena denticola, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans*) in the dental plaque of patients with periodontitis was associated with increased levels of pro-inflammatory cytokines and changes in lymphocyte subsets in peripheral blood. Our data confirm that the lymphocyte count the peripheral blood of periodontitis patients was statistically significantly higher (*P*<0,001) than that of a group of healthy subjects.

IL-4 and IL-5 are Th2 anti-inflammatory cytokines that regulate a number of cell types and their functions. IL-4 is a 14-kDa molecule that is primarily produced by T cells; it has a range of functions, including the co-stimulation of B cells, T cells, and mast cells, and induction of antibody isotype switching to IgE and, in humans, to IgG4 [21,22]. IL-5 exists as a 34-kDa homodimer, is primarily produced by activated T cells, and regulates the production of eosinophils [23]. Recent studies have shown that susceptibility to periodontitis may involve a Th2 response to specific types of periodontal bacteria [21]. The major cytokines produced by Th2 cells are IL-4, IL-5, IL-6 and IL-10, which also assist in antibody

production. IL-4 is considered to be a B cell stimulatory factor that promotes immunoglobulin (Ig) class switching to IgE [24].

Several studies have demonstrated that the localised absence of IL-4 in diseased periodontal tissues is associated with periodontal disease activity and progression [25]. Pradeep et al. [26] reported that the mean concentration of IL-4 in the gingival crevicular fluid was decreased in patients with disease compared with patients in good periodontal health. Changes in cytokine levels, including those of IL-1β, IL-2, IL-4, IL-8 and IFN-γ, were reported in inflamed shallow and deep periodontal sites of patients with generalized chronic and generalized aggressive periodontitis in comparison with the levels of these cytokines in shallow sites from subjects with gingivitis [27]. Taken together, these findings suggest that the localised absence of IL-4 might lead to the progression of gingivitis into periodontitis [28]. IL-4 is very efficient in inhibiting the production of the pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-6, and also of the chemokine IL-8 [29]. With the use of polyclonal monospecific antibodies, it has been shown that Th2 cells are more abundant than Th1 cells in periodontal disease; as a result, IL-4 increases in comparison with IL-2 in periodontitis granulation tissue [30]. These data highlight the likely multifunctional protective role of IL-4 in inflammatory periodontal disease. Lack of IL-4 may result in breakdown of immune function regulation and in enhanced macrophage survival in inflammatory lesions [28].

In the study of Tam et al. [31], both IL-4 and IL-5 were found to be degraded and inactivated by the major virulence factor of P. gingivalis, the RgpA-Kgp complex, under physiologically relevant conditions. These results are consistent with the notion that diffusion of RgpA-Kgp complexes from subgingival dental plaque into the interstitial fluid of gingival tissue results in the degradation and inactivation of at least some of the IL-4 and IL-5 present. IL-4 plays a crucial role in host anti-inflammatory responses. This dysregulation is likely to be localised to the site of infection, where there is a high level of pathogens in subgingival plaque and of secreted proteinases in the subjacent tissues [32]. In the tissues, IL-4 and IL-5, along with other cytokines, may be rapidly degraded, and their bioavailability may be reduced [31]. Using serum samples separated from whole blood, Robati et al. [28] detected an association between generalized aggressive periodontitis, low levels of IL-4 as an anti-inflammatory cytokine, and high levels of IL-6 as a pro-inflammatory cytokine.

Our findings confirm the data of Sonck et al. [33], which indicate that ß-glucan has a beneficial effect. In the present *in vitro* study, different concentration range osf ß-glucan were used to determine whether that compound has a stimulatory effect on neutrophils, monocytes, and lymphocytes. We found that the leukocyte medium stimulated by unopsonised *E. coli* and treated with 0.2 mg or 0.4 mg of ß-1,3/1,6-glucan of patients with periodontitis produced significantly higher (P<0,001; P<0,001) levels of IL-4 and IL-5 than the analogous medium of healthy subjects. The leukocyte count in the incubation medium might not have had any substantial influence on the results for the groups studied.

In a mouse model system, poly-ß1-6-glucotriosyl-ß1-3-glucopyranose glucan significantly enhanced neutrophil migration and bacterial *Porphyromonas gingivalis* reduction, thereby decreasing bacterial infection [34]. Research findings indicate that ß-1,3/1,6-glucan can improve humoral immunity and modulate cellular immunity by mitigating the elevation of pro-inflammatory cytokines, thereby enhancing the increase of anti-inflammatory cytokines through an immunomodulating effect [33].

5. Conclusions

The results obtained in the present *in vitro* study demonstrate that the immunomodulating effects of ß-1,3/1,6 glucan result in a greater increase in cytokine IL-4 and IL-5 production by the leukocyte medium taken from periodontitis patients after stimulation by unopsonised *E. coli* than that observed in the leukocyte medium taken from healthy subjects. This suggests that ß-1,3/1,6 glucan constituents may offer perspectives for the development of a new therapeutic approach to the prevention and treatment of inflammatory periodontal diseases.

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