EXPERIMENTAL INVESTIGATIONS

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Effect of (1→3),(1→6)-*β*-Glucan on In Vitro Production of Cytokines by Leukocytes of Patients With Periodontitis

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Key words: *periodontal disease; (1→3),(1→6)-β-glucan; interleukin 10; tumor necrosis factor α.*

Summary. *Background. The aim of the study was to investigate the effect of (1→3),(1→6)-β-glucan on the production of interleukin 10 (IL-10) and tumor necrosis factor α (TNF-α) in vitro by peripheral blood leukocytes of patients with periodontitis.*

Material and Methods. In total, 20 patients suffering from untreated severe chronic generalized periodontitis were enrolled in this study. Periodontitis was confirmed by clinical and radiologic examination. Besides, 20 periodontally healthy patients served as a control group. Peripheral venous blood was sampled from the patients, and isolated leukocytes were treated with (1→3),(1→6)-β-glucan from yeast at different concentrations. The levels of IL-10 and TNF-α secreted by the leukocytes unstimulated and stimulated with unopsonized E. coli in vitro were determined by the enzyme amplified sensitivity immunoassay method.

Results. Our data showed that (1→3),(1→6)-β-glucan induced a significant decrease (P<0.05) in the TNF-α level and a significant increase (P<0.001) in the IL-10 level in the media of unstimulated and stimulated leukocytes of the patients with periodontitis in comparison with those of the healthy subjects.

Conclusions. The present in vitro study showed that (1→3),(1→6)-β-glucan modulated the response of leukocytes of the patients with periodontitis differently in comparison with those of the healthy subjects. It increased the release of IL-10, which is protective of the tooth-supporting tissues in patients with periodontal disease, but decreased the release of TNF-α, which is mainly responsible for the destruction of the tooth-supporting tissues during periodontal disease.

Introduction

The development of inflammatory periodontal disease is determined by a number of important interrelated factors: the host immuno-inflammatory response, accumulated dental microbial plaque, genetic environmental and conditionally pathogenic periodontal bacteria and their metabolites, etc. (1, 2). The number of leukocytes, which migrate to the gingival sulcus and periodontal pockets, increases with the progression of inflammation. Neutrophils, monocytes/macrophages, T lymphocytes, and B cells accumulate in periodontal lesions, where tissue destruction takes place (3). Microorganisms produce destruction directly through their endotoxins and indirectly through the activation of host cells, which produce biologically active substances, such as cytokines, arachidonic acid metabolites, and proteolytic enzymes (4–6). When exposed to bacteria-derived products or inflammatory mediators, neutrophils respond by chemotaxis, phagocytosis, and microbial killing through oxygen radical and nonoxygen mechanisms (7). Neutrophils with their numerous biologically active substances and secretory granules, which comprise the main part of leukocytes in the gingival crevice, play the most important role in the protection of the macroorganism against periodontal infections (8). The destruction of the tooth-supporting tissues is associated with the release of proteolytic enzymes, collagenolytic matrix metalloproteinases, and reactive oxygen species mainly from neutrophils activated by bacterial lipopolysaccharides (9). The inflammation may depend on constitutional factors modulated by proinflammatory cytokines, such as interleukin 1*β* (IL-1*β*), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor *α* (TNF-*α*), and anti-inflammatory cytokines, such as interleukin 4 and interleukin 10 (IL-10) (10). The main function of IL-10 is the limitation and termination of inflammatory response (11), and it has a key role in maintaining

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health and stability in periodontal tissues (12). On the other hand, TNF-α and IL-1β are important in causing destructive inflammation (5, 13, 14).

β-Glucans, branched glucose polymers derived from the cell wall of a variety of plants and microorganisms, such as barley, yeast, and fungi, are well known as activators of the innate immunity in mice and humans (15). Glucans from yeast and mushrooms are (1→3),(1→6)-β-glucans, while glucans from oat and barley are (1→3),(1→4)-β-glucans. The activation of macrophages, neutrophils, and natural killer cells by β-glucan leads to elevated phagocytic and cytotoxic activities and production of reactive oxygen intermediates and proinflammatory cytokines in vitro and in vivo (16). The immuno-stimulatory effects of β-glucan on leukocytes are mediated by binding to C-type lectin receptor dectin-1, to a lectin domain within the complement receptor type 3 (CR3; CD11b/ CD18, MAC-1), or to toll-like receptors (TLR) (17). Some reports have shown that β-glucans increase the production of TNF-α by the immune cells (18–20); on the other hand, other reports have demonstrated the protective effect of β-glucans on the tissues during inflammatory response, as it suppresses TNF-α secretion (21).

Therefore, we hypothesize that β-glucan can modulate the release of IL-10 and TNF-α from leukocytes and convert it to a pattern, which is more protective of the tooth-supporting tissues. To address the hypothesis, the effect of (1→3),(1→6)-β-glucan from yeast on the production of these cytokines by peripheral venous blood leukocytes of patients with periodontal disease in vitro was investigated.

Material and Methods

Patients

A total of 40 patients (age range, 20–45 years) who were treated in the Clinic of Dental and Maxillofacial Orthopedics, Hospital of Lithuanian University of Health Sciences, were recruited into the study. The subjects were assigned to two groups: healthy subjects (n=20) and patients with periodontitis (n=20). The periodontally healthy subjects served as a control group: they had no pathological dentogingival pockets and no bleeding on probing (PI, <0.02). The patients suffered from untreated severe generalized chronic periodontitis, confirmed clinically and radiographically. The extent of periodontal destruction was determined according to the Russell periodontal index (PI) (22). The patients with periodontitis met the following criteria: minimum 20 remaining teeth, periodontal pocket depth ⩾6 mm on at least 2–3 teeth in a quadrant, and vertical and horizontal bone resorption visible on x-ray. The patients with severe periodontitis (PI, >6.0) were included in this study. The characteristics of the groups are presented in Table. The patients in both groups were free of systemic diseases, were not allergic, and had used no medication within the 6-month period before the study. All the participants were nonsmokers, and women were not pregnant.

Table 1. Characteristics of the Study Groups

Characteristic	Study Group	
	Healthy Subjects n=20	Patients With Periodontitis n=20
Age, years	32.0 (1.6)	32.1 (1.2)
PI score	0.02 (0.01)	6.5 (0.21)*

Values are mean (SD). PI, Russell periodontal index.
*$P<0.001$ vs. healthy subjects.

This study was approved by the Kaunas Regional Bioethics Committee (No. BE-2-76) in accordance with the Declaration of Helsinki. All the subjects involved in this study signed informed consent.

Blood Sampling and Leukocyte Isolation

Fifteen milliliters of peripheral venous blood were taken from the patients before the morning meal. The test tube (containing heparin, 20 IU/mL) was positioned at an angle of 45 degrees and kept for 1 hour at 37°C. Subsequently, the supernatant plasma rich in leukocytes was aspirated, and the leukocyte count was standardized to 1×10^9/L cells with Hank's balanced salt solution (Sigma Chemical Co., St. Louis, Missouri, USA). The cells were counted using a hematological blood analyzer ADVIA 2120 (Siemens Healthcare Diagnostics, Dublin, Ireland). Unstimulated and stimulated leukocytes were used in the study. For stimulation, leukocytes were pretreated with unopsonized *Escherichia coli* (3×10^6 cells/mL) for 45 minutes at 37°C (23). *E. coli* ATCC 25922 was grown in the Laboratory of Microbiology, Lithuanian University of Health Sciences. Samples of the *E. coli* culture for the investigations were used within 24 hours at a concentration of 3×10^6 cells/mL.

β-Glucan

Moreover, (1→3),(1→6)-β-glucan from yeast (Jee Lawson, London, UK) was dissolved in phosphate buffer. Then, 0.025 mL of the (1→3),(1→6)-β-glucan solution at a concentration of 8 mg/mL or 16 mg/mL was added to each test sample in order to have a final β-glucan dose of 0.2 mg or 0.4 mg per each sample, respectively.

Experimental Protocol

The experiments were performed with unstimulated and stimulated leukocytes from the periodontally healthy patients and patients with periodontitis. Each test tube received 0.475 mL of the solution with leukocytes unstimulated or stimulated

by unopsonised *E. coli* (1×10^9/L). Then, 0.025 mL of phosphate buffer (control) or 0.025 mL of the (1→3),(1→6)-*β*-glucan solution at a concentration of 8 mg/mL (0.2 mg per sample, 0.2 mg BG treatment) or 0.025 mL of the (1→3),(1→6)-*β*-glucan solution at a concentration of 16 mg/mL (0.4 mg per sample; 0.4 mg BG treatment) was added to each test tube.

The prepared test samples were placed into a thermostat at 37°C and kept for 5 hours. Then, the levels of IL-10 and TNF-*α* were measured in the media. The experiments with leukocytes of each patient, unstimulated and stimulated by unopsonised *E. coli*, were performed in triplicates.

Determination of Cytokines

The levels of IL-10 and TNF-*α* in the media of the samples with peripheral venous blood leukocytes were determined by the enzyme amplified sensitivity immunoassay (EASIA) method.

Human IL-10 EASIA kits (Biosource Europe S.A., Belgium) and Human TNF-*α* EASIA kits (Biosource Europe S.A., Belgium) were used for the in vitro quantitative determination of IL-10 and TNF-*α* concentrations, respectively, in the media of human leukocytes. For the determination of IL-10 concentration, the enzyme-substrate reaction was terminated by the addition of sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450±2 nm. A standard curve of optical density (OD) versus IL-10 concentration (pg/mL) was computed. The concentration of IL-10 in the samples was then determined by comparing the OD of the samples with the standard curve.

To determine the TNF-*α* level, the bound enzyme-labeled antibody was measured through a chromogenic reaction. A chromogenic solution was added and incubated. The reaction was stopped with the addition of a stop solution, and a microtiter plate was then read at a wavelength of 450±2 nm. The amount of substrate turnover was determined colorimetrically by measuring the absorbance, which is proportional to the TNF-*α* concentration. A calibration curve was plotted, and the TNF-*α* concentration (U/L) in the samples was determined by interpolation from the calibration curve.

Statistical Analysis

SPSS 15 package was used for statistical analysis. Mean and standard deviation were calculated. The level of cytokines between the two groups was tested for a significant difference using the nonparametric Mann-Whitney test. Differences in parameter distribution within the groups were tested using the nonparametric Friedman and Wilcoxon signed rank tests. Differences were considered significant when $P<0.05$.

Results

The measurement of the level of cytokines was performed in the media of leukocytes harvested from the patients with untreated chronic generalized periodontitis and from the subjects with intact periodontal tissues after the immunomodulatory effect of (1→3),(1→6)-*β*-glucan from yeast at different concentrations in vitro.

Our data showed that the levels of both TNF-*α* and IL-10, released by unstimulated control leukocytes from the patients with periodontitis and those of the healthy subjects, did not differ significantly ($P>0.05$) (Figs. 1 and 2). In vitro treatment with (1→3),(1→6)-*β*-glucan produced an increase in the IL-10 level and a decrease in the TNF-*α* level in the media of leukocytes from the patients with periodontitis, but only the dose of 0.4 mg showed a significant effect ($P<0.001$) in comparison with untreated leukocytes. In contrast, there was no effect of (1→3),(1→6)-*β*-glucan on the production of these cytokines by unstimulated leukocytes from the healthy subjects with intact periodontium (Figs. 1 and 2).

Stimulation of leukocytes with unopsonized *E. coli* of the patients with periodontitis induced a significantly different production of IL-10 and TNF-*α* in comparison with the healthy subjects in all the treatment groups (Figs. 1 and 2). Before the treatment with *β*-glucan, the TNF-*α* level was higher ($P<0.001$) in the media of stimulated leukocytes from the patients with periodontitis in comparison with the healthy subjects (Fig. 1). After the treatment with (1→3),(1→6)-*β*-glucan, the TNF-*α* level decreased more considerably ($P<0.001$) in the leukocytes of the patients with periodontitis than in those of the healthy subjects. Both 0.2-mg and 0.4-mg doses of (1→3),(1→6)-*β*-glucan were effective in the treatment of stimulated leukocytes in comparison with control in both groups of the patients ($P<0.001$ and $P<0.05$, respectively) (Fig. 1). A larger dose (0.4 mg) of (1→3),(1→6)-*β*-glucan was more effective in decreasing the release of TNF-*α* ($P<0.001$) by leukocytes of the patients with periodontitis, when only a slight but significant decrease of the TNF-*α* level ($P<0.001$) was detected with stimulated leukocytes of the healthy subjects (Fig. 1).

The level of IL-10 was also significantly higher ($P<0.001$) in the media of stimulated control leukocytes of the patients with periodontitis in comparison with the healthy subjects (Fig. 2). In vitro treatment of leukocytes of the patients with periodontitis with (1→3),(1→6)-*β*-glucan significantly ($P<0.001$) increased the level of cytokines, but a larger dose of (1→3),(1→6)-*β*-glucan was more effective ($P<0.001$) (Fig. 2). In contrast, the IL-10 level in the media of stimulated leukocytes of the healthy subjects was slightly increased ($P<0.001$) by (1→3),(1→6)-*β*-glucan at a dose of 0.4 mg only.

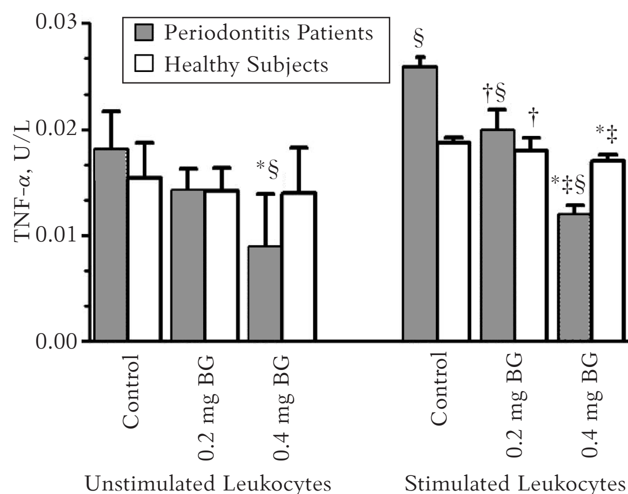

Fig. 1. The levels of tumor necrosis factor α (TNF-α) produced by the leukocytes unstimulated and stimulated by unopsonized *E. coli* and those treated with (1→3),(1→6)-β-glucan (BG) from yeast in vitro

The bars represent the mean TNF-α concentration (SD) in U/L detected in the media of human leukocytes (control) and those treated with (1→3),(1→6)-β-glucan from yeast at a dose of 0.4 mg/per sample (0.4 mg BG) or 0.2 mg/per sample (0.2 mg BG). Human leukocytes were stimulated by unopsonized *E. coli*. *$P<0.001$, †$P<0.05$ vs. control; ‡$P<0.001$ vs. treatment with 0.2 mg BG; Friedman and Wilcoxon signed rank tests. §$P<0.05$ patients with periodontitis vs. healthy subjects; Mann-Whitney test.

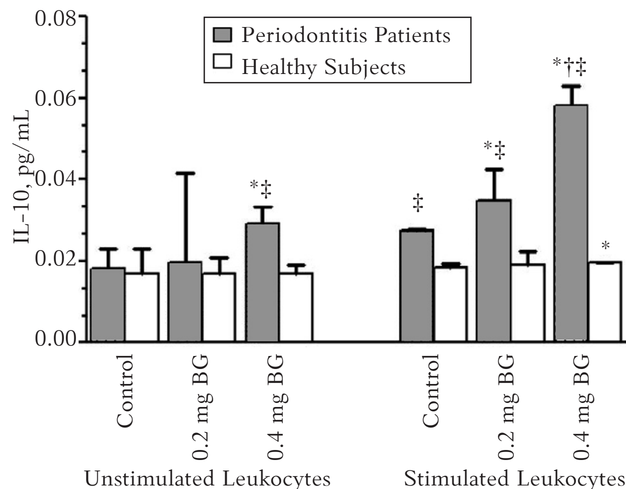

Fig. 2. The levels of interleukin 10 (IL-10) produced by the leukocytes unstimulated and stimulated by unopsonized *E. coli* and those treated with (1→3),(1→6)-β-glucan (BG) from yeast in vitro

The bars represent the mean IL-10 concentration (SD) in pg/mL detected in the media of human leukocytes (control) and those treated with (1→3),(1→6)-β-glucan from yeast at a dose of 0.4 mg/per sample (0.4 mg BG) or 0.2 mg/per sample (0.2 mg BG). Human leukocytes were stimulated by unopsonized *E. coli*. *$P<0.001$ vs. control; †$P<0.001$ vs. treatment with 0.2 mg BG; Friedman and Wilcoxon signed rank tests. ‡$P<0.05$ patients with periodontitis vs. healthy subjects; Mann-Whitney test.

Discussion

Recent studies have proved that dental plaque causes an inflammatory periodontal disease (24). The immune response is an important factor in the etiopathogenesis of this condition. Released granule components from infiltrating leukocytes, such as lysosomal enzymes and reactive oxygen species, which are normally intended to degrade ingested microbes, can also lead to tissue destruction and amplification of the inflammatory response (25). It has been suggested that hyperresponsiveness of monocytes to the products of dental plaque, especially the endotoxin of gram-negative bacteria and the secretion of a high level of proinflammatory cytokines, may have a role in the pathogenesis of periodontal disease (26). Both IL-10 and TNF-α act conversely and are considered key factors in antibacterial immune responses (27). It was thought that IL-10 inhibits cytokine synthesis, but recent data have been more supportive of its immune-regulatory role. IL-10 is also known for its capacity to suppress monocyte differentiation to antigen-presenting cells, inhibit the expression of most inducible chemokines involved in inflammation, inhibit the production of prostaglandin E_2 (PGE_2), and enhance the production of anti-inflammatory mediators; other stimulatory effects of IL-10 include the enhancement of proliferation and differentiation of B cells and their maturation into plasma cells (27).

TNF-α enhances osteoclast formation and activity, induction of matrix metalloproteinase expression, and stimulation of PGE_2 production. These events cause the destruction of connective tissue and bone. The use of TNF-α antagonists results in the reduction of periodontal inflammation, indicating a cause-effect relationship between TNF-α and periodontal infection (13).

Some authors have reported that peripheral blood neutrophils in periodontitis show both hyperreactivity to dental plaque organisms and hyperactivity in unstimulated generation to release reactive oxygen species and granule content (28, 29). Our data agree with this as stimulated leukocytes from the patients with periodontitis produced a significantly higher level of TNF-α ($P<0.001$) and a significantly higher level of IL-10 ($P<0.001$) than analogous cells of the healthy subjects. In a susceptible host, the overreaction of the host response occurs resulting in the excessive production of proinflammatory cytokines, such as IL-1, TNF-α, and IL-6, and in the subsequent loss of periodontal attachment (27). The ability of IL-10 to inhibit directly the production of TNF-α also affects several steps in antimicrobial immunity (11). TNF-α and IL-1β are critical determinants of the progression of periodontitis. These two primary cytokines can induce the expression of adhesion molecules and secondary mediators that facilitate and amplify the inflamma-

tory response, matrix metalloproteinase production, and bone resorption (9, 14). Therefore, the suppression of TNF-α release and an increase in IL-10 production may protect tissues in the focus of inflammation (21).

Sontck et al. (30) have indicated that different β-glucans have a stimulatory effect on neutrophils, monocytes, and lymphocytes in vitro. Binding of β-glucans to their receptors stimulates intracellular signaling pathways with the following activation, translocation, and nuclear binding of immuno-regulatory and proinflammatory transcriptional activator proteins (31). Some studies have shown that β-glucans are able to activate leukocytes, stimulating the production of proinflammatory mediators, such as IL-1, IL-6, TNF-α, IFN-α, and IL-8 (27, 30, 32), or convert a Th2-type response to a Th1-type response that favors the activity of cytotoxic T lymphocytes (33). In contrast, other studies have reported that the administration of β-glucan, isolated from *Phellinus linteus*, results in a decrease of TNF-α, IL-12, and IL-1β levels, and it may contribute to the long survival of mice during septic shock (34). Significantly enhanced neutrophil emigration and reduction of *Porphyromonas gingivalis* following the administration of poly-β-1-6-glucotriosyl-β-1-3-glucopyranose glucan in mice (35), and reduced infection-stimulated bone resorption in rats have been shown as well (36).

Our current data confirmed the immunomodulatory activity of (1→3),(1→6)-β-glucan from yeast on human leukocytes in vitro. It decreased the release of TNF-α and increased the release of IL-10 significantly ($P<0.001$) from the leukocytes stimulated by bacteria of the patients with periodontitis, whereas slight changes in the production of these cytokines were observed in the leukocytes of the healthy patients. It may contribute to hyperreactivity and hyperactivity of leukocytes of patients with periodontitis, as other authors report (28). Moreover, a more potent effect was achieved with the leukocytes affected by bacteria, i.e., with stimulated leukocytes. Our data showed that (1→3),(1→6)-β-glucan influenced the secretion of cytokines in a dose-dependent manner, which agrees with other reports (21, 34). The results of our study support an idea that β-glucan can modulate destructive inflammation by its capability to suppress TNF-α production induced by periodontopathogenic bacteria and their toxins (27). On the other hand, β-glucan increases the release of IL-10, which is considered as an immunoregulator, and can prevent hyperinflammatory processes (11).

Conclusions

The present in vitro study showed that (1→3),(1→6)-β-glucan modulated the response of the leukocytes of the patients with periodontitis differently in comparison with those of the healthy subjects. It increased the release of IL-10, which is protective of the tooth-supporting tissues in patients with periodontal disease, but decreased the release of TNF-α, which is mainly responsible for the destruction of the tooth-supporting tissues during periodontal disease.

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Statement of Conflict of Interest

The authors state no conflict of interest.

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