Journal of Clinical Periodontology

Soluble β -1,3/1,6-glucan from yeast inhibits experimental periodontal disease in Wistar rats

Breivik T, Opstad PK, Engstad R, Gundersen G, Gjermo P, Preus H. Soluble β -1,3/ 1,6-glucan from yeast inhibits experimental periodontal disease in Wistar rats. J Clin Periodontol 2005; 32: 347–352. doi: 10.1111/j.1600-051X.2005.00672.x. © Blackwell Munksgaard, 2005.

Abstract

Objective: We have investigated whether a purified immunomodulatory water soluble β -1,3/1,6-glucan isolated from the cell wall of Bakers yeast, *Saccharomyces cerevisiae*, would influence the progression of ligature-induced periodontal disease, and to modulate accompanying cytokine and hypothalamic–pituitary–adrenal (HPA) axis responses to a lipopolysaccharide (LPS) challenge.

Material and Methods: β -1,3/1,6-glucan (10 mg/kg/day) was given in the drinking water to Wistar rats during the entire experiment, starting 14 days before disease induction, while control rats were given tap water only. Periodontal disease was assessed when the ligatures had been in place for 35 days.

Results: Orally administered soluble β -1,3/1,6-glucan significantly reduced periodontal bone loss as measured on digital X-rays (p = 0,026). Glucan-treated rats also showed a significantly enhanced plasma level of the HPA axis-driven hormone corticosterone (p = 0.047), and of the cytokine transforming growth factor-1 β (p = 0.032), as well as a tendency to enhanced IL-10 (p = 0.106), induced by intraperitoneally administered LPS.

Conclusion: Soluble β -1,3/1,6-glucan administered by the oral route diminishes ligature-induced periodontal bone loss in this model. This effect may be attributable to the well documented ability of β -1,3/1,6-glucan to stimulate macrophage phagocytosis and to skew the T helper (Th)1/Th2 balance towards Th1 and T regulatory responses. The HPA axis may play a significant role in β -1,3/1,6-glucan induced immune modulation.

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Key words: β -1,3/1,6-glucan; cytokines; hypothalamo–pituitary–adrenal axis; periodontal disease; macrophages

Accepted for publication 21 July 2004

Periodontal disease is a tissue destructive immune and inflammatory condition triggered by increased colonization of pathogenic microorganisms, termed periodontopathogens, in sub-gingival dental plaques. The disease leads to destruction of the tooth supporting tissues, including destruction of periodontal attachment fibres and resorption of the alveolar bone. The disease may progress to periodontal pocket formation, increased tooth mobility and tooth loss in the most severe cases (Lindhe 1995).

The increased colonization of perio dontopathogens, including some anaerobic Gram-negative species, motile rods, spirochetes, as well as virus (Socransky & Haffajee 1992, Sela 2001, Kamma & Slots 2003) may be a result of a too weak specific T helper 1 (Th1)-mediated immune response (Wassenaar et al. 1998, Bartova et al. 2000, Breivik & Thrane 2001), while the periodontal fibre destruction may be caused by reactive oxygen species (ROS) and matrix metallo-proteinases (MMPs) released from immune system cells belonging to the innate immune system, such as polymorphonuclear phagocytes (PMNs) when they fight and destroy periodontopathogens (Birkedal-Hansen 1993, Ding et al. 1997, Lohinai et al. 1998). The bone resorption by osteoclast seems to be a secondary process that prevents the bone from being infected (Breivik & Thrane 2001). Thus, in patients with periodontal disease, the immune response may be biased towards a too strong T-helper 2 (Th2) mediated immunity, and immune responses that accelerate cell-mediated immunity, which include macrophage activation and Th1 dominance, as well as T regulatory (T_{reg}) responses, may inhibit the tissue destruction observed in periodontal disease.

Severe periodontal disease has been found to be associated with genetics,

aging, and environmental factors, including smoking, poorly controlled diabetes, and poorly developed psychological coping strategies to traumatic life events. such as the loss of a spouse by death (Breivik & Thrane 2001, Hugoson et al. 2002). All these factors, which predispose to periodontal disease, are associated with increased activation of the hypothalamic-pituitary-adrenal (HPA) axis (Breivik & Thrane 2001), which is one of the major overarching immunoregulatory mechanisms controlled by the brain (Ader et al. 1995, Chrousos 1995, Breivik et al. 1996). HPA axis activation by pathogenic microorganisms or other danger signals, and the subsequent release of glucocorticoids (predominantly cortisol in man and corticosterone in rodents), are known to down-regulate cell-mediated immunity and bias immune responses towards Th2 and T regulatory (Treg) responses (Rook 1999, Chen et al. 2004), partly by downregulating IL-12 and increasing IL-10 release from antigen-presenting cells (Vieira et al. 1998). Thus, selected periodontal risk factors may predispose to disease development and progression by their effect on the HPA axis (Breivik & Thrane 2001).

This relationship between HPA axis reactivity, immune and glucocorticoid responses, and periodontal disease has been demonstrated clearly in a rat model (Breivik et al. 2000a, b, c, 2001a, b, 2002a, b). Individuals that exhibit circadian dysregulation of HPA axis, and thus show inappropriate glucocorticoid responses (whether it is genetically determined, age-related, or environmentally stressinduced) to inflammatory signals, may be more susceptible to periodontal disease. For example, postnatal treatment that permanently modulates subsequent excitability of the HPA axis lead to corresponding changes in immune responses and susceptibility to periodontal disease (Breivik et al. 2002b). Thus, treatment strategies that could lead to restoration of a proper balance between Th1, Th2 and Treg responses, which have been generated when the immunity has been affected by immune-suppressing factors, may be favourable for the treatment of periodontal disease. This effect has recently demonstrated by vaccination with an immunomodulatory agent developed from killed Mycobacterium vaccae (Breivik & Rook 2000, 2002, 2003).

 β -1,3-glucan is a polyglucose structure in the cell wall of fungi, certain bacteria and plants, and has be found to have immunomodulatory effects in animals and humans (Williams et al. 1996). For example, it enhances immune function by activating macrophages and establishes Th1 dominance, which induces cell-mediated immunity (Inoue et al. 2002, Lee et al. 2002). Moreover, β -glucan enhances resistance to infections caused by bacteria and parasites, certain tumours, as well as stimulates wound healing (Brown & Gorden 2001, Yun et al. 2003).

Inability to mount a suitable immune and stress response to control the colonization of pathogenic microorganisms in sub-gingival dental plaques may play a significant role in periodontal disease development and progression (Breivik et al. 1996, Breivik & Thrane 2001). A consequential objective for periodontal research would thus be to find a well-tolerated substance that can stimulate protective immune responses and thus control and prevent the growth of periodontopathogens. In this study we have used a purified soluble carbohydrate polymer known as β -1,6-branched-1,3-glucan extract from Bakers yeast (Saccharomyces cerevisiae), which has been demonstrated to be a potent inducer of innate immune mechanisms both in animal models as well as in human cell cultures (Engstad 1994, Engstad et al. 2002).

The purpose of this study was to examine whether soluble β -1,3/1,6-glucan could influence the tissue destruction in a well-established experimental model of periodontal disease in Wistar rats.

Materials and Methods

Animals

Thirty male Wistar rats, weighing 260–300 g, were obtained from Möllegaard Breeding Center (Ejby, Denmark), and used after 2 weeks of acclimatization. Standard rat chow pellets and tap water were available *ad libitum*. The animals were housed in groups of five under a 12/24 h light/ dark cycle (light on 7:00 14:00 hours) with temperature and humidity at 22°C and 40–60%, respectively, and grouped in two at random. The experiments were registered and approved by the Norwegian Experimental Animal Board (NEAB).

Soluble β -1,6-glucan and treatment of rats

Water soluble β -1,3/1,6-glucan was provided by Biotec Pharmacon ASA (Tromsø, Norway) at a concentration of 2% in water (pH 4.5) and stored at +4°C. The β -1,3/1,6-glucan (10 mg/kg/ day) was administered in the drinking water to 15 rats, starting 14 days before application of the ligatures. Fifteen control rats received tap water only.

Experimental periodontal disease

Fourteen days after β .1,3/1,6-glucan induction, all animals were anaesthetised by subcutaneous injection in the neck with Hypnorm-Dormicum (fenatyl/fluanizone, midazolam), 0.2 ml/100 g body weight. Two rats in each group died during the anaesthesia. A sterile silk ligature (Ethicon Perma-hand[®] size 3/0, Norderstedt, Germany) was tied around the neck of the maxillary right 2nd molar tooth in the gingival sulcus. The ligature served as a retention device for oral microorganisms. The left 2nd molar served as a non-ligated internal control tooth. Thirty-five days after application of the ligatures, all animals were killed by decapitation. The maxillae were excised and fixed in 4% formaldehyde.

Radiographic examination

The specimens were stabilised with dental wax on a Sidexis digital X-ray sensor, orientated with the axis of the teeth parallel to the sensor surface by using $\times 4$ magnification loupe glasses (Zeiss, Aalen, Germany). The distance between the cemento-enamel junction (CEJ) and bone (B) on mesial and distal surfaces of the 2nd molars were displayed digitally. The examiner was unaware whether the specimens came from experimental or control animals. Each X-ray was read three times, and the mean of the three readings calculated. The reliability of the method has been tested earlier (Breivik & Rook 2000). The average percentage difference between individual readings and the mean of the respective triplicate was $3.48 \pm 5.12\%$.

Lipopolysaccharide (LPS) challenge

The peripheral blood monocytes of humans with periodontal disease release

Table 1. Differences in weight, ligature-induced alue	plar bone loss, and glucocorticoid hormor	ne (corticosterone) and cytokine	responses to LPS in
β-glucan and tap water drinking Wistar rats			

	Treatment		Mann-Whitney,
	β -glucan ($n = 13$)	Tap water $(n = 13)$	<i>p</i> -value
Weight at glucan induction (g)	269.8 ± 12.9	267.8 ± 9.8	0.659
Weight at ligature placement (g)	318.4 ± 20.8	322.2 ± 20.0	0.750
Weight at sacrifice (g)	388.6 ± 30.0	382.5 ± 29.3	0.601
Weight change (g)	118.6 ± 21.8	114.7 ± 24.1	0.649
Bone loss, X-ray (mm)	0.92 ± 0.10	1.03 ± 0.09	0.016
Corticosterone (nm/l-2h after i.p. LPS, day of sacrifice)	1371.00 ± 308.0	1067.4 ± 421.6	0.047
TGF-1 β (pg/ml 2 h after i.p. LPS, day of sacrifice)	34.04 ± 5.83	27.78 ± 8.02	0.032
IL-10 (pg/ml 2h after i.p. LPS, day of sacrifice)	70.00 ± 30.02	51.54 ± 25.79	0.106
TNF- α (pg/ml 2h after i.p. LPS, day of sacrifice)	3513.54 ± 3400.77	3733.23 ± 4766.91	0.890

All data are shown as means \pm standard deviation.

TGF-1 β , transforming growth factor-1 β ; IL-10, interleukin-10; TNF- α , tumour necrosis factor- α ; i.p., intraperitoneally; LPS, lipopolysaccharide.



Fig. 1. Digital X-rays of maxillary right upper molar teeth in Wistar rats illustrating differences in alveolar bone loss in orally β -glucan treated (left picture) and control rats (right picture) given tap water only.

an altered profile of mediators when stimulated by LPS (Fokkema et al. 2002), and the immune and HPA systems are mutually regulatory (Turnbull & Rivier 1999, Eskandari & Sternberg 2002). The animals were therefore injected with LPS (*Escherichia coli* serotype 0111:B4, Sigma Chemical, St. Louis, MO, USA) shortly (2 h) before ending the experiment to assess whether the treatment regime influenced on the corticosterone response to LPS.

After decapitation of the rats, the blood samples were collected (6–10 ml from each animal) in vacutainer tubes (10 ml without additives) and allowed to clot on ice for 1 h. Thereafter, the samples were centrifuged for 20 min at $2000 \times g$, and the serum samples removed, and stored at -20° C prior to analysis of corticosterone and cytokines.

Assay of corticosterone

Corticosterone was measured with ¹²⁵I radioimmunoassay (RIA) coat-A-count kit from Diagnostic Products Corporation (Los Angeles, CA, USA), catalog number TKRC1. The detection limit was 5.7 ng/ml.

Assay of serum IL-10, TGF-1 β and TNF- α

The levels of IL-10, TGF-1 β , and TNF- α in the serum samples were measured by means of enzyme-linked immunosorbent assay (ELISA) kits from R&D systems, Inc. (MN, USA), with catalogue numbers R1000, MB100 and RTA00, respectively. The minimum detectable dose for IL-10 and TGF-1 β is less than 31.2 pg/ml, and less than 12.5 pg/ml for TNF- α .

Statistics

Values are expressed as mean \pm Std., and differences between the β -glucantreated rats and control rats were examined using Student's *t*-test. Because some of the data were not normally distributed, the non-parametric Mann–Whitney test was used for between-group comparisons.

Results

The treatment had no effect on the weight of the animals. The controls and glucan-treated animals weighed 319 ± 20.8 and 322.2 ± 23.0 g, respectively, when the ligatures were applied, and 388.6 ± 30.0 and 382.5 ± 29.3 g,

respectively, at the termination of the experiment, 35 days later.

The animals were sacrificed 35 days after application of the ligature. Radiographically the mean bone loss measured as the distance between the cemento-enamel junction (CEJ) and the most coronal bone (CEJ-B) in the experimental sites in Wistar rats that had been treated with oral β -glucan 12 days before application of the ligature was 0.94 ± 0.11 mm. In the control rats the same distance was $1.05 \pm 0.12 \,\mathrm{mm}$ (Table 1). The bone loss in the treatment group was significantly reduced compared with that seen in the untreated controls (p = 0.026, Student's t-test)(Fig. 1).

Treatment with oral β -glucan lead to a significantly stronger corticosterone response 2h after LPS injection (glucan-treated $1371.0 \pm 308.0 \text{ nm/l}$; controls $1067.4 \pm 421.6 \text{ nm/l}; p = 0.0047;$ Table 1). The glucan-treated rats showed a higher TGF-1 β (34.04 ± 5.83 pg/ml) compared with controls $(27.78 \pm 8.02 \text{ pg/ml}; p = 0.032)$, and a tendency to a higher serum level of IL-10 (70.00 \pm 30.02 pg/ml) compared with controls $(51.54 \pm 25.79 \text{ pg/ml})$; p = 0.106). There was no difference between the groups in TNF- α plasma levels $(3513.54 \pm 3400.77 \text{ pg/ml} \text{ versus})$ $3733.23 \pm 4766.91 \text{ pg/ml}; p = 0.89$).

Discussion

The present study shows that orally administrated soluble β -1,3/1,6-glucan resulted in enhanced resistance to ligature-induced periodontal disease in Wistar rats. In addition, the β -glucan-treated animals showed an increased HPA axis and TGF-1 β responses to a robust LPS challenge, as well as a

tendency to stronger IL-10 response. There are several possible modes of action that could explain these effects.

 β -1,3/1,6-glucan is known to act as an immunostimulant enhancing hostmediated immune responses to pathogens as well as tumour cells, especially by activating macrophages, and stimulating differentiation of T-cells towards the Th 1 cellular subset (Brown & Gorden 2001, Suzuki et al., 2001). These effects are likely to be mediated by Toll-like receptors (TLRs) and the newly discovered Dectin-1 receptor (Brown et al. 2003). These cell surface pattern recognition receptors, which are highly expressed on dendritic cells and also on macrophages, lead to stimulation of cytokine production such as interleukin (IL)-12 and tumour necrosis factor- α (TNF- α) (Gantner et al. 2003). The cytokines (together with a number of other immune signals) orchestrate an immune and inflammatory response by Th cells that skew the Th1/Th2 balance towards a Th1-dominated response (Suzuki et al. 2001). For example, β -glucan stimulates TNF- α production and macrophage phagocytosis (Lee et al. 2002), and switches the balance from IgG1 antibodies (which are Th2-dependent antibody subclasses) towards a Th1-dependent IgG2a response (Suzuki et al. 2001). β -glucan also induces the production of Th1-stimulating cytokine interferon- γ (IFN- γ), but suppresses IL-4 that is inducing Th2 responses (Inoue et al. 2002). Experiments also show that β glucan can modify immune responses by stimulating the production of the Th1skewing cytokine IL-12 versus IL-6, IL-10 and prostaglandin E2 (PGE2) by macrophages (Murata et al. 2002).

In addition, soluble β -glucan has been found to alter the immune response to LPS as shown by suppression of the production of the cytokine TNF- α from monocytes and lymphocytes stimulated by LPS (Soltys & Quinn 1999). LPS consists of potent antigens and immunostimulators located on the outer membrane on all Gramnegative bacteria. In addition, soluble β -glucan protects against LPS-induced shock in rats (Rasmussen & Seljelid 1991, Vereschagin et al. 1998). This can be explained by its capability to suppress LPS-induced TNF- α production. The immune response to β -glucan has further been shown to be different from the response to LPS. The latter causes high recruitment of PMNs (Thorn et al. 2001), whereas β -glucan primarily

seems to induce recruitment of macrophages (Jørgensen et al. 1993).

The ability of β -glucan to stimulate macrophages may in part explain the result found in this experiment. In the gingival tissues from patients with untreated advanced periodontitis, there is an apparent failure of the recruitment and activation of macrophages compared with that found in gingival tissues of patients with gingivitis (Chaple et al. 1998). Moreover, the cytokine pattern in gingival tissue from cases of periodontal disease reveals a relative increase in Th2 cytokines (IL-4, IL-5 and IL-13) whether studied by in situ hybridization (Tokoro et al. 1997) or immunohistochemistry (Yamazaki et al. 1995). Increased expression of IL-13 has been a consistent finding whereas IL-4 mRNA has been elusive (Fujihashi et al. 1996, Yamamoto et al. 1997, Takeichi et al. 2000). However, difficulties in demonstrating mRNA encoding IL-4 by RT-PCR are not unusual because of its extremely low mRNA copy number, and the very short half-life of this mRNA (Breivik & Rook 2003). The presence of a Th2-biased response to the periodontopathogens is further supported by the observation that peripheral blood cells of patients, or T-cell clones derived from them, release more Th2 cytokines in response to periodontopathogens in vitro (Wassenaar et al. 1998, Bartova et al. 2000). Interestingly, the peripheral blood monocytes of patients with periodontal disease release less IL-12 and more PGE2 in response to LPS than do monocytes from normal controls, and this is a shift that would drive responses to periodontopathogens towards Th2 (Fokkema et al. 2002).

It is also significant that the factors that predispose to periodontal disease, such as smoking (Martinez-Canut et al. 1995), age (Burt 1994), diabetes (Thorstensson & Hugoson 1993), depressive mood stated caused by experiences of negative life events and poor psychological coping (Hugoson et al. 2002), all lead to increased secretion of glucocorticoids (Breivik & Thrane 2001), which are known to bias responses towards Th2 (Ramirez et al. 1996, Vieira et al. 1998, Visser et al. 1998). This relationship between HPA axis activation, glucocorticoid activity and periodontal disease has been demonstrated clearly in the rat model (Breivik et al. 2000a, b, c, 2001a, b, 2002a, b).

In this experiment, β -1,3/1,6-glucan showed a significant protective effect on

the development of ligature-induced periodontitis and simultaneously resulted in a stronger HPA axis response to an acute and robust LPS challenge, as demonstrated by higher corticosterone levels. At the first glance, this result may appear contradictory. However, the HPA axis serves as a peripheral limb via which the brain is suppressing proinflammatory Th1 responses, while boosting anti-inflammatory Th2 responses (Elenkov & Chrousos 1999). There are also indications that immuneinduced HPA activation may skew Th1 and Th2 effector responses towards T_{reg} responses (Chen et al. 2004), leading to stronger IL-10 and TGF- β cytokine responses (McGuirk & Mills 2002). Thus, the continuous β -1,3/1,6-glucan treatment may have lead to an enhanced HPA axis response in order to control the Th1 responses, and these rats may be more capable of suppressing LPSinduced immune responses. The immunomodulatory effects of β -glucans on LPS challenges may, thus, in part be a result of its effect on the HPA axis.

In conclusion, these data suggest that the enhanced periodontal disease resistance induced by the soluble β -1,3/1,6glucan treatment, could be explained by its action on the immune system and the HPA axis. The relationships between HPA axis activation and immunity, tissue destruction induced by immune mediators, and periodontal disease development and progression are complex. However, some of these issues can be addressed by further experimental animal studies, and β -1,3/1,6-glucan would be an obvious candidate to be tested as a therapeutic agent in clinical trials for periodontal disease.

Acknowledgements

We are grateful to the Norwegian Defence Research Establishment, Division of Protection and Material, Kjeller, Norway, for supporting this work.

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