Abstract. Aim: β-Glucan is one of the most abundant polymers in nature and has been established as an immunomodulator. This compound has notable physiological effects on mammalian immune systems, including anti-tumor and anti-infective activities and can activate the immune response. It is considered that the immune-stimulating activities of β-glucan can depend on physicochemical parameters, such as molecular size. Saccharomyces cerevisiae, also known as baker’s yeast, is a frequently used source of β-glucan. The aim of the experiments was to investigate how different Saccharomyces cerevisiae β-glucan preparations with different molecular size affect interferon-gamma (IFN-γ) production in BALB/c mice. Materials and Methods: In vivo and in vitro BALB/c mouse models were used for the investigations. Different β-glucan preparations were orally administrated in the in vivo experiments. IFN-γ production in BALB/c mice was analyzed by enzyme-linked immunosorbent assay and measuring interferon-γ RNA concentration. Results: The results showed that orally-administered β-glucan from S. cerevisiae enhanced IFN-γ production in BALB/c mice in the in vivo model, but not by mouse leukocytes in vitro. Moreover, water-soluble β-glucan enhanced IFN-γ production more effectively than did particulate β-glucan. Conclusion: IFN-γ plays an important role in immunity against viral and bacterial infections. Our experiments have shown that β-glucan preparations enhance IFN-γ production in BALB/c mice and can be potentially used for immune system stimulation in mammals. Current results may be used to develop soluble β-glucan nutritional supplements.

β-Glucan is one of the most abundant polymers in nature. It is composed of repeating glucose monomer units which are linked by β-1,3-glycosidic bonds. β-Glucan is produced by a variety of different organisms – bacteria, fungi, and plants (1). Most commonly, these polymers are found in the cell wall of microorganisms and in cereals (1, 2). There exist many different types of this polymer, which differ in their structure and complexity. For instance, β-glucan from baker’s yeast, Saccharomyces cerevisiae, predominantly consists of the β-1,3-linked backbone having branches via β-1,6-linkages and is insoluble in water; β-glucan from Agrobacterium biobar, curdlan, is a linear β-1,3-glucan which forms elastic gels upon heating in aqueous suspension; and cereal β-glucans are often β-1-3,1-4-glucans and many are soluble in water (1, 3, 4). Polymers linked to β-1,3 glicosidic bonds have a notable physiological effect on mammalian immune systems, can activate the immune response via immune cells, and trigger the immune response (2, 5-7). Thus β-glucan can be potentially used in the treatment of the different infections and can possibly increase immune cell cytotoxicity for tumor cells (1, 2, 8). The immune-stimulating activities of β-glucans can depend on their physicochemical parameters, such as their solubility and molecular weight (9, 10).

S. cerevisiae is a frequently used source of β-1,3-glucan (2, 8, 11-14). A large part of the fungal cell mass consists of β-glucans, which are major structural components of various yeast cells and vital for cell functions (1, 6, 14). Recent studies have shown that yeast cell wall β-glucan, whether soluble or particulate, can exhibit antimicrobial and antitumor effects (10, 12). It is believed that β-glucan can be used as a safe, effective, therapeutic or prophylactic agent, either alone or as adjuvant, to enhance or prime the immune response in mammals with normal or decreased immunological function (12). Particulate β-glucan preparations derived from S. cerevisiae are already widely used as nutritional supplements (11).
Current data suggest that β-glucan can affect both innate and adaptive immunity (10). One of the major effects of immune system stimulation is a secretion of cytokines, which are primarily involved in host responses to disease or infection (15). Interferon-gamma (IFNγ) is a dimerized soluble cytokine mainly produced by lymphocytes and has broad effects on the immune system; it has immunomodulatory, anti-microbial, and anti-proliferative activities and modulates the production of several other cytokines and chemokines (16, 17). One of the most important effects of IFNγ is the activation of the anti-microbial functions of macrophages. When activated by IFNγ, macrophages exhibit increased pinocytosis, phagocytosis and enhanced microbial killing ability. Moreover, IFNγ up-regulates cell-surface class I Major Histocompatibility Complex (MHC) and increases the potential for cytotoxic T-cell recognition of antigens, which is important for the host response to intracellular pathogens. IFNγ also up-regulates the class II antigen-presenting pathway and promotes peptide-specific activation of CD4+ cells (18).

Some reports have shown that β-glucans from different sources can elicit IFNγ production in vitro and in vivo in mammals (9, 17, 19, 20). Hashimoto et al. determined that clinically-applied β-glucan sonifiran from S. spheroides increased IFNγ concentration in serum and spleen in AKR/N, C3H/HeN and C3H/HeJ, ICR and WBB6F1-W/WV mice (16). In addition, researchers found an increased IFNγ concentration in the spleen of BALB/c mice (9). Budak et al. showed that administration of S. cerevisiae particulate β-glucan increased IFNγ concentration in serum and spleen in AKR/N, C3H/HeN, C3H/HeJ, ICR and WBB6F1-W/WV mice (16). Moreover, IFNγ up-regulates cell-surface class I Major Histocompatibility Complex (MHC) and increases the potential for cytotoxic T-cell recognition of antigens, which is important for the host response to intracellular pathogens. IFNγ also up-regulates the class II antigen-presenting pathway and promotes peptide-specific activation of CD4+ cells (18).

Materials and Methods

Chemicals. All processes and reactions were performed using reagent-grade materials. S. cerevisiae IHEM 7071 was purchased from The Belgian Coordinated Collections of Microorganisms (BCCM, Brussels, Brussels-Capital Region, Belgium). Insoluble whole β-glucan particles from S. cerevisiae cell wall were extracted according to the method published by Javmen et al. with some modifications (14). Briefly, 3g of dry baker’s yeast was enzymatically lysed by Streptomyces rugerensis (JSC Biocentras, Vilnius, Vilnius County, Lithuania) yeast lysing preparation and washed with distilled water. Yeast cell walls were isolated by centrifugation and resuspended in 1 M NaOH solution (1:5 w/v). Then the mixture was heated to 100˚C with stirring for 2 h. Whole β-glucan particles were harvested by centrifugation and washed with distilled water until reaching a neutral pH. Soluble β-glucan preparations were made by enzymatic hydrolysis of whole glucan particles using S. rugerensis β-glucanase and fractionation with a Sephacryl S-200 column (3). Six β-glucan preparations were made in this way: one insoluble whole β-glucan particle preparation and five soluble preparations with different molecular sizes (from largest to smallest: S1, S2, S3, S4 and S5).

Mice and treatment. Female, 8-week-old mice, with body weight of 20-24 g, were kindly provided by the Animal Facility of Centre for Innovative Medicine, Vilnius, Lithuania. Experimental conditions were in compliance with Good Laboratory Practices and with the Law of the Republic of Lithuania on the Care, Keeping and Use of Animals as well as secondary legislation (approval no. 0202). The animals were acclimatized for one week before the study; they were housed under constant conditions of temperature, humidity and with a 12 h/12 hlight/dark cycle. Commercial pellet diet and fresh drinking water were provided ad libitum.

In vivo IFNγ induction experiment: Experimental groups (5 mice/group) were fed daily with 0.1 ml water containing 0.1 mg β-glucan for one week. Control mice (5 mice) were fed in the same way as the experimental mice, but without β-glucan. A blood sample (100 μl) was taken from the tail vein of each mouse on days 2 and 7 after the start of β-glucan administration. A blood sample was also taken on day 14 after the end of β-glucan administration (three weeks after the start of the experiment). IFNγ concentration was determined by measuring IFNγ RNA in the blood samples.

In vitro IFNγ induction experiment: Leukocytes from the blood of five control (untreated) female mice were isolated by RBC lysis buffer protocol for mouse blood (Alfa Aesar GmbH & Co KG, Karlsruhe, Baden-Württemberg, Germany). The blood was taken from Jugular vein. White blood cells (WBCs; 1x10^5/ml) were stimulated in a 24-well plate with different β-glucan preparations (10 μg/ml); control WBCs were: i) stimulated with Lipo polysaccharides (LPS) from Escherichia coli (Sigma-Aldrich Co. LLC, St. Louis, Missouri, United States) (1 μg/ml); ii) without stimulation. WBCs were incubated in a humidified incubator with 5% CO2 at 37˚C for 48 h. After incubation, the culture supernatants were harvested and assayed for IFNγ by ELISA.

Determination of IFNγ RNA concentration. The total RNA from 20 μl of mouse blood samples was extracted using QIAamp® RNA Blood Mini kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer’s instructions. Elution was performed with 20 μl RNase-free water included in the kit.

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Real-time one-step Reverse transcription polymerase chain reaction (RT-PCR) was performed using Rotor-Gene Q 5-plex model (QIAGEN). Rotor-Gene Q Series Software 1.7 version was used for the process. Each 15 μl reaction contained 200 nM of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 100 nM of each probe (Integrated DNA Technologies), 6 μl of RNA and Verso 1-Step qRT-PCR Kit components (7.5 μl of 2×1-Step qPCR Mix, 0.75 μl of RT Enhancer, 0.15 μl of Verso Enzyme Mix; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Designed primer pairs, probes and conditions of amplification are provided in Table I.

The 2^-ΔΔCT method was applied for the evaluation of relative gene expression data. Mouse Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was used for data normalization.

**IFNγ ELISA assay.** IFNγ concentration in leukocyte supernatants was determined using the Mouse IFN gamma ELISA Kit (Thermo Scientific, Waltham, Massachusetts, USA); sensitivity: ≤2 pg/ml, limit of detection: 8 pg/mL.

**Statistical analysis.** The significance of differences between the means was assessed using Student’s t-test.

### Results

**Formulation of β-glucan preparations of different molecular sizes.** We extracted insoluble β-glucan from *S. cerevisiae* of baker’s yeast. Then we hydrolyzed insoluble β-glucan using β-glucanase (3). Reaction products from β-glucan particle hydrolysis were fractionated with Sephacryl S-200. Fractions with different size β-glucan molecules were collected and analyzed with a Zetasizer Nano device to investigate the size of the β-glucan molecules (3). We determined that the sizes of the soluble β-glucan molecules were ~0.7-23 nm (Figure 1). A further five soluble β-glucan preparations were made; molecular sizes of these preparations were approximately: S1 ~19-23 nm; S2 ~15-18 nm; S3 ~11-14 nm; S4 ~8-10 nm; S5 ~3-7 nm; oligomers, whose molecular size was less than 3 nm, were not analyzed because of their small dimensions.

**IFNγ synthesis induction in vivo.** Five soluble β-glucan preparations and insoluble β-glucan particle suspension (0.1 mg/day) were orally administered to BALB/c mice. IFNγ concentration in the blood samples was measured after two and seven days during β-glucan administration and two weeks after the final oral treatment. Only two soluble β-glucan preparations, S1 and S3, induced the release of statistically significant amounts of IFNγ after two days of β-glucan administration compared to control animals, and almost all soluble preparations (except S4) statistically significantly induced the release of IFNγ after seven days’ administration (Figure 2). Insoluble whole β-glucan particle preparation and S4 preparations induced IFNγ production in a similar manner to other soluble preparations, but the result was not statistically significant according to Student’s t-test. We also measured IFNγ concentration in the blood of mice on day 14 after the final oral treatment: the IFNγ concentration in all mice had returned to the level of the control animals.

**Induction of IFNγ synthesis in vitro.** Mouse leukocytes were isolated from BALB/c mice and treated with β-glucan preparations (10 mg/ml). IFNγ concentration was measured after 48 h incubation with different preparations. As seen in Figure 3, the results of the in vitro treatment were partially opposite to those of the in vivo experiment.

Leukocytes treatment with LPS and preparation S4 had no effect on IFNγ. Other β-glucan preparations in reduced IFNγ concentration in the cell supernatant to different extents. There was a statistically significant difference (according to Student’s t-test) in IFNγ concentration between the leukocyte supernatants which were treated with S1, S2, and S3 preparations and cells without treatment. The differences in IFNγ concentration in supernatants from cells treated with insoluble preparation and S5 compared with untreated cells were not statistically significant, but had the same tendency for decreasing IFNγ concentration as after treatment with S1, S2 and S3.

**Discussion**

*S. cerevisiae* β-glucan is an established immunomodulator (9, 10, 12, 21). β-Glucan belongs to a group of physiologically active materials, which are called biological
response modifiers (22). Many studies have demonstrated that various forms of β-glucan exhibit antibacterial and antitumor activities (8, 9, 10, 23, 24). This is the main reason why S. cerevisiae is broadly investigated. β-Glucan can also act as a ligand for different immune receptors (2, 6). Hence the nature of the immune response can depend on the size of β-glucan molecule. Therefore, we investigated how different sized β-glucan molecules from S. cerevisiae affect the production of the important immune system cytokine, IFNγ, in mouse models in vivo and in vitro.

As described above, β-glucan preparations induced IFNγ production in BALB/c mice in vivo. Hence, β-glucan can activate IFNγ production, but we did not find a positive result in the in vitro model with BALB/c mouse blood leukocytes; conversely, leukocyte treatment with some soluble preparations (S1, S2 and S3) actually reduced the IFNγ concentration in the supernatant. These results might be explained in the following way: despite the fact that lymphocytes (T-cells, natural killer cells and others) are the main IFNγ producers, and lymphocytes make up ~70% of all leukocytes in blood from BALB/c mice, lymphocytes cannot be activated to produce cytokines in vitro without the assistance of the other immune system components. This hypothesis can also be confirmed by the fact that IFNγ production is controlled by other cytokines secreted by antigen-presenting cells, most notably interleukins (IL) 12 and 18. For instance, Interleukin-12 (IL12) is produced by macrophages after the recognition of pathogens, and in our in vitro model, there were no macrophages. Moreover, β-glucan receptors are in the main carried on monocytes/macrophages, neutrophils and eosinophils, but not on T- or B-lymphocytes and only natural killer lymphocytes abundantly produce β-glucan receptors (25). We can conclude that β-glucan activates IFNγ production in BALB/c mice, but there is no direct lymphocyte cell activation by β-
Our investigations revealed that orally administered β-glucan from *S. cerevisiae* enhances IFNγ production in BALB/c mice *in vivo*, but not by mouse leukocytes *in vitro*. Soluble β-glucan enhances IFNγ production more effectively than particulate β-glucan. β-Glucan molecules with different molecular size have different effects on IFNγ production *in vivo*. The current results may be used to develop soluble β-glucan nutritional supplements.

**References**


3. Javmen A, Grigiškis S, Rudenkov M and Mauricas M: Oral administration of β-glucan from *S. cerevisiae* enhances IFNγ production in BALB/c mice *in vivo*, but not by mouse leukocytes *in vitro*. Soluble β-glucan enhances IFNγ production more effectively than particulate β-glucan. β-Glucan molecules with different molecular size have different effects on IFNγ production *in vivo*. The current results may be used to develop soluble β-glucan nutritional supplements.


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