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Research

The Optimum Dose of Beta-Glucan for Stimulating Peripheral Blood Mononuclear Cells (PBMCs) to Produce Cytokines: In Vitro Study

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ABSTRACT

Background: Beta-glucan has been frequently used in laboratory trials as an immunomodulator in both in vivo and in vitro studies, but the effective dose for measuring its performance has not been established. Like other immunomodulators, researchers must establish the right dose of beta-glucan in their laboratory experiments for the purpose of testing substances as immunomodulators without achieving false-positive or –negative results. This study aimed to determine the optimum dose of beta-glucan to induce cytokine production by peripheral blood mononuclear cells (PBMCs) in vitro.

Subjects and Method: This was a laboratory experimental study. This study measured the production of two cytokines, including interferon gamma (IFN-γ) and interleukin 12 (IL-12), from the isolated PBMCs of healthy subjects. The doses of beta-glucan used as immunomodulator included 1, 5, 10, 20, and 50 µg/ml. Beta-glucan was added to the PBMC culture medium, and the PBMCs were cultured for 6 days. On the sixth day, the supernatant was harvested and the cytokine production was analyzed using sandwich enzyme-linked immunosorbent assay (ELISA). Cytokines were also analyzed using the human IFN-γ ELISA kit and the human IL-12 ELISA kit, and data analysis was performed by one-way ANOVA.

Results: IFN-γ levels were found to be increased in the group treated with 5 µg/ml beta-glucan. The highest IFN-γ levels (70.0 pg/ml) were observed in the group treated with 10 µg/ml beta-glucan. The production of IL-12 increased sharply in the group treated with5 µg/ml beta-glucan but decreased in the group treated with 10 µg/ml beta-glucan. The mean cytokine levels of the beta-glucan group were found to be significantly different from those of the control group (p=0.001). One-way ANOVA revealed that the highest IL-12 production (77.2 pg/ml) occurred at a dose of 5 µg/ml beta-glucan. This average value was significantly different from the average production of IL-12 in the control group (p=0.001).

Conclusion: The optimum dose of beta-glucan for stimulating PBMCs to produce IFN-γ in vitro was 10 µg/ml, while for the production of IL-12, the dose was 5 µg/ml. Both cytokines can be measured within 6 days of cell culture.

Keywords: beta-glucan, immunomodulator, cytokines

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BACKGROUND

Cytokines are protein molecules secreted by cells and are involved in intercellular communication, acting as mediators to enhance the immune response through interaction with specific cell surface receptors in leukocytes (Peters, 1996). Immune cells must produce enough cytokines to keep the body heal-

thy in cases of both the invasion of pathogenic microorganisms and the occurrence of cell malignancy processes. The occurrence of genetic defects causes less cytokine production, due to which the body becomes susceptible to disease (Ramirez-Alejo and Santos-Argumedo, 2014; Bustamantea et al., 2014; Caragol and Casanova, 2003). The primary cytokines that play an important role in fighting bacterial infections are interferon gamma (IFN-γ) and interleukin 12 (IL-12) (Lauw, 2000). IL-12 is a regulatory cytokine that activates natural killer (NK) cells and T helper 1 (Th1) cells to produce IFN-γ (Budak et al., 2008). Meanwhile, IFN-γ itself plays an important role in activating the process of phagocytosis and improves protection against intracellular bacterial infections by increasing proactive reactive oxygen species (ROS) (Marchi et al., 2014).

Various substances are known to act as immunomodulators that can stimulate cytokine production. One of the substances used as an immunomodulator is beta-glucan. Utilization of beta-glucan as an immunomodulator developed along with the progress in the concept of complementary alternative medicine or modalities (CAM), which began to develop again in the early 2000s. The research on medicinal mushrooms as a complementary therapy has been widely increasing along with the development of CAM application in the society (Shah et al., 2011). Beta-glucan can be extracted from various living things such as yeast, mushroom, barley, and wheat, (Noss et al., 2013; Limberger-Bayer et al., 2014; Goncalves et al., 2014) and it has become one of the substances that has been intensively studied as an immunomodulator in various diseases caused by malignancy or infection processes (Yun et al., 2003; Chan et al., 2009).

Beta-glucan has been used in laboratory experiments as an immunomodulator in both in vivo and in vitro studies (Javmen et al., 2015, but the effective dose has not yet been determined to measure its performance. Researchers have used different doses of beta-glucan in their experiments. Like other immunomodulators, it is necessary to establish an appropriate dose for use in laboratory experiments, so that the research objectives can be achieved without positive-false or -negative results.

Therefore, the aims of this study were to determine the optimum dose of beta-glucan to induce cytokine production by peripheral blood mononuclear cells (PBMCs) in vitro and to provide basic research data and a scientific basis for its application in laboratory assessment.

SUBJECTS AND METHOD

1. Study Design

This was a laboratory experimental study that compared the effects occurring in the treatment group and the control group.

2. Population and Sample

In this study, PBMCs isolated from healthy donors were used as the research subject. The inclusion criteria of the subjects were as follows: healthy men or women aged 18–25 years, showing negative test results for HIV, hepatitis B, and hepatitis C screening (conducted at RSU Banyumanik Semarang), and not taking immunosuppressant or immunomodulators. Before participation in the study, the subjects were briefed about the study and agreed to be involved in the research by signing the informed consent form.

3. Study Variables

The dependent variable was cytokines production. The independent variable were doses of beta-glucan for Stimulating PBMCs.

4. Operational Definition of Variables

Isolation and Culture of PBMCs were carried out according to the following steps. A total of 4 ml of peripheral blood of subjects was collected into a heparin lithium tube (Endo Vacu Tube) and allowed to stand for

60 min at room temperature. The tube was then centrifuged at 1500 rpm for 15 min. Then, Ficoll (Amersham Ficoll-Paque PLUS) was added to the tube and centrifuged at 1500 rpm for 15 min. The buffy coat was collected and washed twice with PBS. In the PBMC tube, 1 ml of complete RPMI medium was added, and the cells were counted under a microscope using a hemocytometer. PBMCs(5×105/ml) were cultured in 24-well plates containing the complete RPMI medium as the control group and in plates containing the RPMI medium added with beta-glucan at various doses, including 1, 5, 10, 20, and 50 µg/ml, as the treatment groups. Subsequently, the cells were incubated in a 5% CO2 incubator at 37°C for 6 days.

Beta-Glucanof manufactured baker's yeast Saccharomyces cerevisiae was used. Before use, beta-glucan was dissolved in ultrapure water and vortexed for 15 min.

Measurement of Cytokines (IFN-γ and IL-12) were assessed in this study. On the sixth day, the supernatant was collected and the cytokine production was determined using sandwich enzyme-linked immunosorbent assay (ELISA). The cytokines were further analyzed using the human IFN-γ ELISA kit and the human IL-12 ELISA kit according to the manufacturer's instructions. The collected supernatant was centrifuged at 1000 rpm for 20 min. Before use, the plate was rinsed twice. The standard and a sample of 100 µl were collected into each well and incubated at 37°C for 90 min. Furthermore, 100 µl of biotin-detection antibody working solution was added to each well and incubated at 37°C for 60 min. The plate was washed three times. Into each well of the plate, 100 µl of SABC working solution was added and incubated at 37°C for 30 min. The washing step was performed five times. Then, 90 µl of TMB substrate was added and incubated for 15 min at 37°C. In the next step, 50 µl of stop solution was added, and the cytokine levels were read on a micro plate reader at an optical density (OD) of 450 nm absorbance.

5. Data Analysis

The normality of data was assessed using the Kolmogorov–Smirnov test, while the comparison between treatments was done using one-way ANOVA. Data were displayed and analyzed using Microsoft Excel 2010 and IBM SPSS Statistics 20, with the significance level set at $p < 0.05$.

6. Research Ethic

This study was conducted with the approval of the Committee on Ethical Research for Health of the Faculty of Medicine, Diponegoro University, and the Kariadi Hospital, Semarang, Indonesia. Ethical clearance for this project is available under the registration no. 467/EC/FK-RSDK/VII/2017 at the secretariat of the Committee on Ethical Research for Health of the Faculty of Medicine, Diponegoro University, and the Kariadi Hospital. Laboratory tests were conducted at the Parasitology Laboratory of the Faculty of Medicine, Gadjah Mada University, Yogyakarta, from March to April 2018.

RESULTS

The production of IFN-γ and IL-12 was measured in the six PBMC groups, including the control group and the groups treated with 1, 5, 10, 20, and 50 µg/ml of beta-glucan. Figure 1 shows the results of IFN-γ and IL-12 measurements for the treatment and the control groups. IFN-γ levels were increased in the PBMC group cultured with the addition of beta-glucan from the dose of 5 µg/ml till the dose of 10 µg/ml, at which the highest IFN-γ level was detected. In addition, at the subsequent dose (20 µg/ml), the IFN-γ level began to decrease. The PBMC group treated with 50 µg/ml beta-glucan also showed a decrease in IFN-γ level.

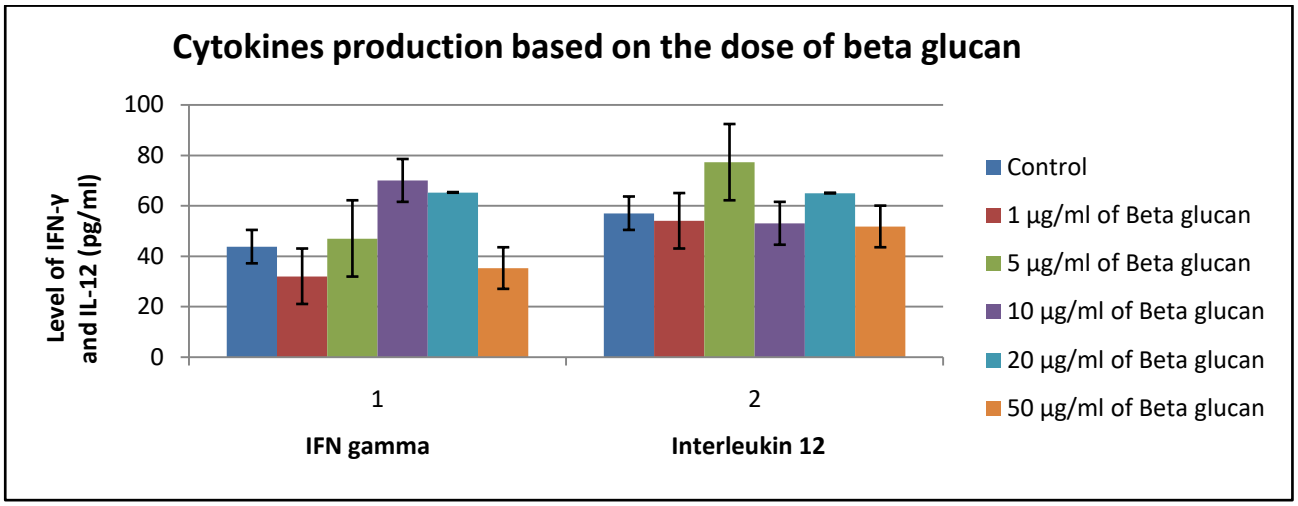

Figure 1. Cytokine production by PBMCs in control group and treatment groups

Meanwhile, the highest level of IL-12 production was detected in the group treated with5 µg/ml beta-glucan. In the PBMC cultures treated with 10 µg/ml beta-glucan, the IL-12 production was decreased, but at the dose of 20 µg/ml beta-glucan, the IL-12 levels started to elevate. However, at the dose of 50 µg/ml, the levels of IL-12 decreased again.To determine the optimum dose of beta-glucan to be used as an immunomodulator, the normality was assessed using the Kolmogorov–Smirnov test, which showed that both IFN-γ and IL-12 data were normally distributed. Furthermore, one-way ANOVA was conducted to determine the difference in the mean production of both cytokines under various treatments. The results of both tests are presented in Table 1.

Table 1. The mean distribution of IFN-γ and IL-12 data according to the dose of beta-glucan

Variable	Dose of beta-glucan (µg/ml)	Mean (pg/ml)	SD	p
IFN-γ	0	43.7	0.35	0.001
	1	32.0	4.24	
	5	47.0	5.65	
	10	70.0	1.41	
	20	65.2	1.76	
	50	35.2	1.06	
IL-12	0	57.0	2.28	0.001
	1	54.0	1.41	
	5	77.2	1.76	
	10	53.0	2.12	
	20	65.0	1.41	
	50	51.7	2.47	

The ANOVA showed that the highest IL-12 production (77.2 pg/ml) occurred at the dose of 5 µg/ml beta-glucan. This average value was significantly different from the mean value of IL-12 production in the control group (p= 0.001).

DISCUSSION

The concept of CAM describes that biologically based therapies use typically natural substances and include herbs and essential oils, special diets, nutritional and food supplements, and other products (Kramlich, 2014) such as beta-glucan. Along with the CAM

concept, the results of this study show that the beta-glucan extracted from the yeast *S. cerevisiae* can be used as an immunemodulator, particularly acting as a stimulator of IFN-γ and IL-12 production in vitro. There are various types of glucan molecules that can be extracted from various sources, including mushrooms, yeast, wheat, and other plants. The potential of glucan as an immunomodulator is determined by its structure and molecular weight (Choromanska et al., 2018).In this study, the beta glucan used as immunomodulator was derived from *S. cerevisiae* because of the structure of β- (1,3) -(1,6) -glucan and molecular weight (35–5000 kDa), this glucan indicates its potential to be used as an immunomodulator, particularly to induce the production of cytokines by immune cells (Noss et al., 2013).

Certain substances can affect the physiology of the body at the right dosage. Administering substances in quantities that are too small cannot produce the expected effect, but administering excessive quantity will also have an impact on the occurrence of toxicity to targeted cells or organs. To obtain the right dose according to the expected goals, researchers must test various doses repeatedly, especially for new substances that are not yet widely known. Several previous studies have shown that the potential of beta-glucan as an immunomodulator depends on the given dose. Some of the cytokines that can be stimulated by beta-glucan include IFN-γ, IL-12 (Budak, 2008), TNF-α, IL-6, and IL-8 (Noss et al., 2013).

In this study, compared with the control group and other beta-glucan doses, the highest production of IFN-γ was observed in PBMCs treated with 10 µg/ml beta-glucan for 6 days, whereas the highest production of IL-12 was detected in PBMCs treated with 5 µg/ml beta-glucan for 6 days. These results are slightly different from those of previous studies using a single dose of 5 µg/ml beta-glucan to obtain the immunomodulator effects of PBMCs on both IFN-γ and IL-12 production (Budak et al., 2008).

Beta-glucan is administered at different doses and times according to the desired goal. In rats infected with influenza virus, beta-glucan may reduce the risk of death from infection at a dose of 881.5 µg/day for 12 days by preventing massive weight loss, stimulating the ability of phagocytosis by mouse peripheral blood neutrophils, and killing by NK cells, as well as by increasing the production of several cytokines such as IFN-γ, IL-1β, and TNF-α (Vitvicka et al., 2015). Other experiments were performed on mice with polymicrobial infection. After 1 h of infection, mice were administered 10 mg/kg of body weight of PGG glucan. After 6 h, the cytokines were measured in blood samples. Results showed that the PGG glucan enhanced the survival in female mice over a 10-day period, but survival in males was improved for only 24 h. In female mice, PGG glucan reduced the levels of IL-6 and IL-10 and also reduced the bacterial burden in the liver (Newsome et al., 2011).

In addition to its use to boost the immune host system during infection, beta-glucan is often used to enhance the immune system in malignancy. Beta-glucan extracted from *S. cerevisiae* has growth inhibitory effects against the murine mammary adenocarcinoma AMN-3 cell line depending on time and concentration. The highest effect was obtained with the beta-glucan extract at higher concentrations after 48 h of exposure in the AMN-3 cell line (Jabber et al., 2011).

In patients with prostate cancer who were administered 20mg of soluble beta-glucan in the form of a carboxymethylated (CM-G) capsule daily for 28 days, the total leukocyte count increased significantly ($p= 0.02$), with no associated changes in the lifestyle habits of the patients. A significant increase ($p=0.001$) was also observed in red

blood cell, hematocrit, hemoglobin, and platelet counts. No changes were observed in the hepatic or renal function after CM-G administration, and there were also no side effects associated with its use (Magnani et al., 2010).

In addition to improving the fitness of immune cells so that they can function optimally, one of the mechanisms of beta-glucan to help the body fight against malignancy is by activating immune responses to induce cell apoptosis through the caspase-3-dependent signaling pathway and to inhibit cell proliferation possibly via the p53-dependent signaling pathway in vivo. Besides, it has been reported that LNT inhibited angiogenesis by suppressing VEGF expression, leading to slow progression of tumors (Xu et al., 2016).

This study demonstrated that the global cytokine production in the beta-glucan group was increased significantly ($p<0.05$). The optimum dose of beta-glucan for stimulating PBMCs to produce IFN-γ in vitro was 10 µg/ml, while it was 5 µg/ml to induce the production of IL-12 by PBMCs. Both cytokines can be measured on the sixth day of cell culture. These findings suggest that beta-glucan is useful as a complementary or adjuvant therapy for improving the production of cytokines.

AUTHOR CONTRIBUTION

Meira Erawati conducted the study, collected the data, analyzed the results, and wrote the article.

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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