SHORT COMMUNICATION

Immunomodulatory Effect of β-Glucan on Peritoneal Macrophages of Babl/c Mice

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A b s t r a c t

We assessed the effect of β-Glucan on macrophages by Griess reagent and viability by MTT assay and cytotoxicity. Assay of macrophages culture supernatants were carried out on WEHI-164 fibrosarcoma cell line as tumor necrosis factor-α bioassay were done. NO release was increased at the dose of 10 μg/ml (P = 0.001) of β-Glucan while the viability of macrophages in all concentrations was the same. In TNF-α bioassay, the supernatant of macrophages stimulated with β-Glucan had a significant cytotoxic effect on WEHI-164 cells (P = 0.023). β-Glucan had a positive effect on increasing tumoricidal activity of macrophages which may help in anti-cancer immune responses.

K e y   w o r d s: β-Glucan, macrophage, nitric oxide, TNF-α

One of the most promising alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses (Nemunaitis, 1997). Several types of immunomodulators have been identified, including mammalian mediators such as interferon-gamma (IFN-γ), granulocyte colony-stimulating factor (Hamilten and Anderson, 2004) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Plaulsen, 2001), as well as the substances isolated and purified from microorganisms (Nemunaitis, 1997). Recently, polysaccharides isolated from botanical sources (mushrooms, algae, lichens and higher plants) (Wasser, 2002) have also attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and their relatively low toxicity (Chihara, 1992). Several major substances with immunomodulatory and/or antitumor activity such as β-D-glucans, polysaccharopeptides (PSP), polysaccharide proteins and proteins have been isolated from mushrooms (Cristina *et al.*, 2005). β-Glucan is a major structural component of fungi and it has reported that fungi have a β-Glucan rich cell wall compromised of glucose residues arranged in β (1–3) D glucopyranosyl polymers with β (1–6) D glucopyranosyl side chains of varying length and frequency distribution that can activate macrophage for release inflammatory agent (Lebron *et al.*, 2003). These extracts exert their biological effect through different mechanisms. One of the active compounds responsible for the immunomodulatory effects of natural products is in the form of complex polysaccharides known as β-Glucans (Chang *et al.*, 2009). Indeed, some the basic mechanisms of the immunostimulatory, anti-tumor, bactericidal and other therapeutic effects of botanical polysaccharides is thought to occur via macrophage stimulation (Wang *et al.*, 1997) and modulation of the complement system (Beutler, 2004). Macrophages represent the first line of host defense. In addition, macrophages can function as antigen-presenting cells and interact with T lymphocytes to modulate the adaptive immune responses (Lingen, 2001). Furthermore, macrophages are involved in tissue remodeling during embryogenesis, wound repair (Klimp *et al.*, 2002), clearance of apoptotic cells and hematopoiesis (Gruchalla and Jones, 2005). Activated macrophages play a critical role in infections by eliminating microbial pathogens through the generation of nitric oxide (NO) (Fang and Vazquez-Torres, 2002). NO as a critical effector molecule of macrophages can be released upon stimulation of macrophages with a variety of stimuli such as bacterial

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products or cytokines. TNF-α also is a main cytokine of macrophages with defined activities (Lorsbach and Russel, 1992). In the present study, we evaluated the immunomodulatory effects of β-Glucan on peritoneal macrophages as NO production and cytotoxic effect of β-Glucan on cancer cell line with emphasis on coordinate macrophage effect on tumor cells and its function as TNF-α bioassay. With this background, β-Glucan was purchased from Sigma as a powder that was purified from *Saccharomycess servisiae*. In this study, we prepared different concentrations of β-Glucan by dissolving the powder in distilled water and these were used in our study. Female inbred BALB/c mice (at 8 to 10 weeks of age) were obtained from Pasteur Institute (Tehran, Iran). All mice were maintained in a specific pathogen-free animal facility at Tarbiat Modares University sterilized water and autoclaved standard mouse pellet throughout the study. The animal study was approved by a local ethics committee. Peritoneal exudates macrophages were harvested by peritoneal lavage from 5 mice by i.p. injection of 10 ml sterile ice cold RPMI-1640 (Sigma Chemical Co). The pooled cells were centrifuged at $200 \times g$, washed with PBS (pH = 7.2) and resuspended in complete RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 u/Penicillin and 100 μg/ml streptomycin and 3×10^5 cells/well were added onto 96-well flat-bottomed plates (Nunc) followed by incubation at 37°C for 4 h under humidified 5% CO_2 atmosphere. The non-adhering cells were then removed by washing the wells three times with PBS. The adherent cells were incubated for desired time cultured in complete RPMI medium and different concentration of β-Glucan (1, 10, 100, 200 μg/ml) was added to macrophage culture as triplicate wells with the final volume of 200 μl/well. Unstimulated macrophages and also macrophages stimulated with 50 IU/ml IFN-γ were considered as negative and positive controls, respectively (Ribeiro-Dias *et al.*, 1998). The cultured cells were incubated at 37°C for 48 h under humidified 5% CO_2 atmosphere. Supernatant fluids from macrophage cultures were collected at the end of incubation time and stored at –20°C for further assays. For MTT assay Macrophage viability was evaluated by the MTT (3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay. After 48 h of macrophage culture, 20 μl of MTT (5 mg/ml in PBS) was added to wells and the plates were incubated for 4 h. Formazan crystals developed from MTT reduction by living cells. The supernatants were then gently removed, 100 μl of isopropanol in 0.04% HCl (Sigma, USA) was added in order to dissolve the formazan crystals. The plates were incubated overnight and the absorbance of each well was measured by ELISA reader (Multiskan MS, England) at wavelength of 540 nm (Ribeiro-Dias *et al.*, 1998). Stimulation Index (SI) was

determined as: Absorbance at 540 nm of Test /absorbance at 540 nm of Control. In case of Measurement of Nitrite Concentration; NO released into the supernatants of cultured macrophages. NO is unstable and rapidly converts to nitrite and nitrate. Accordingly, we estimated the level of NO synthesis by macrophages via measuring the amount of nitrite accumulating in the cultures, using the method of Stuehr and Nathan (1989). Briefly, nitrite concentration was determined with the standard Griess reaction, by adding 50 μl of test solution (supernatants of macrophage culture) to 96-well flat-bottomed plates containing 50 μl of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl) ethylenediamine dihydrochloride/2.5% H_3PO_4] (Merck). The samples were assessed in triplicate. After 15 min at room temperature, the absorbance of each well was measured using a ELISA reader (Multiskan MS, England) microplate reader at 540 nm and the nitrite concentration was determined from a standard curve of sodium nitrite (Stuehr and Nathan, 1989). Mean of nitrite concentration (μM) was expressed. For TNF-α bioassay; Effect of β-Glucan on tumoricidal activity of macrophages in cell-free specimens of each macrophage culture was determined by means of the viability of WEHI 164 mouse fibrosarcoma cell line through MTT assay (Arora *et al.*, 2005). Briefly, macrophages treated with β-Glucan (1, 10, 100 and 200 μg/ml) for 48 h and the supernatant was stored. Then WEHI 164 target cells (2×10^4 cells in 50 μl) cultured in 96-well microtiter plate together with 50 μl of stored culture fluid of treated macrophages treated with β-Glucan (1, 10, 100 and 200 μg/ml). WEHI cultures were incubated for 20 h at 37°C and 5% CO_2 in a humidified incubator. After this time, 10 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for a further 4 h. Then, supernatants were removed from each well and replaced with 100 μl of 0.04 N HCl in isopropanol. The plates were then stored overnight in the dark at room temperature. After dissolving the dark blue formazan, the optical density of each well was measured with an ELISA reader (Multiskan MS, England), using the wavelength of 540 nm. The percentage of target cells death was calculated as Suppression Index (SuI) as: Absorbance at 540 nm of Test /absorbance at 540 nm of Control (Espevik and Nissen-Meyer, 1987). After test were done for Statistical analysis; the cells were harvested from 5 mice and assays were done as triplicate manner. Data was expressed as mean ± SD. The results of MTT test were analyzed, using One-Way Analysis of Variance (ANOVA) followed by Tukey tests, and a value of P < 0.05 were used as the significance levels. After preparation of different concentrations of β-Glucan with dissolving in distilled water doses of 1, 10, 100 and 200 μg/ml of β-Glucan were added to each well of microplate containing macrophage cultures in complete
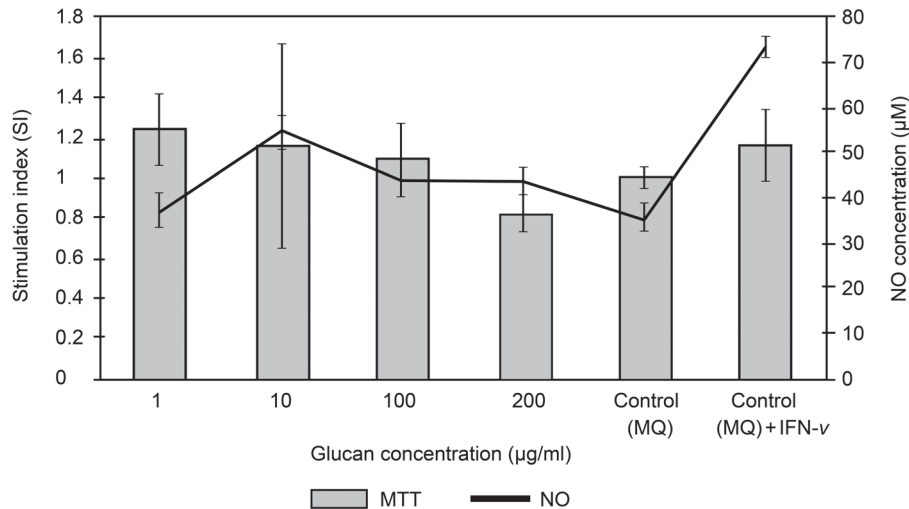
Fig. 1. Effect of β-Glucan on NO release and macrophages viability

Isolated peritoneal macrophages from BALB/c mice were incubated in the absence or presence of β-Glucan for 48 h. The cell supernatants were collected and nitrite production was evaluated. NO production by macrophages was increased at the dose of 10 µg/ml (p = 0.0017) compared to controls. MTT reduction ability of macrophages, as a criteria of cell viability for all concentration of β-Glucan was the same as control (p > 0.05). MQ: Macrophages, IFN: Interferon-gamma. Data represent mean ± S.D

medium. NO amount was evaluated in the collected supernatants of culture. As shown in Fig. 1, NO production was significantly increased ($P = 0.0017$) at the dose of 10 µg/ml compared to the negative control (only macrophages). Results of MTT assay indicated that viability of macrophages in all concentration of Glucan was the same as untreated macrophages (P > 0.05). For evaluate the cytotoxic effect of the supernatant of macrophages stimulated by β-Glucan on the tumor cell line WEHI-164 murine fibrosarcoma as TNF-α bioassay, colorimetric MTT cytotoxicity assay was done. The results (Fig. 2) indicated that the supernatant of treated macrophage contained cytotoxic activity in all

examined doses (P < 0.05). These effects were declared by reduced Suppression Index in β-Glucan-treated group compared to negative control. Cytotoxic effect in IFN-γ positive control group also was significant (P < 0.05). For description of the findings; Glucans are a heterogeneous group of glucose polymers with naturally polysaccharides component consisting of a backbone of β(1,3)-linked β-D-glucopyranosyl units with β(1,6)-linked side chains of varying distribution and length that are produced by a variety of plants, such as oat, barley, and fungi (Lebron et al., 2003). β-Glucans are the constituents of the cell wall of certain pathogenic bacteria and fungi. The main components of the
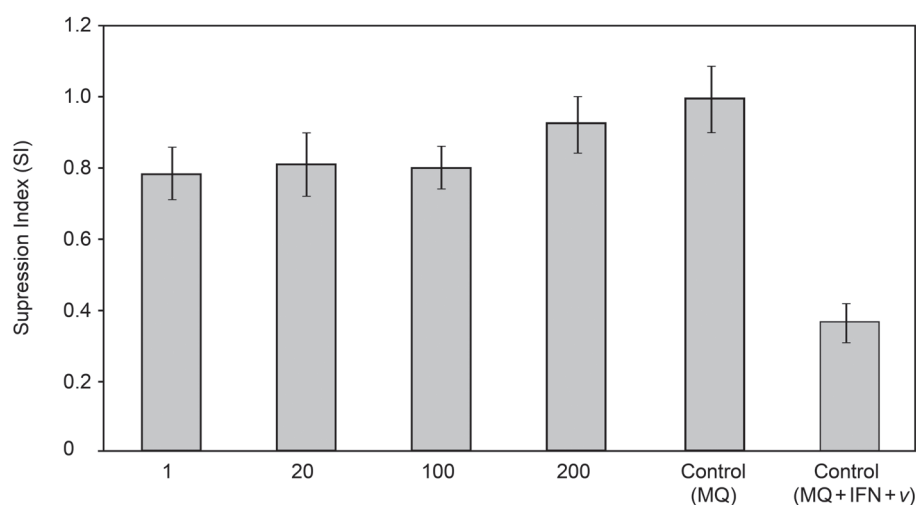

Fig. 2. Mean ±SD of Suppression Index (SuI) for cytotoxic effect of macrophage supernatant on WEHI-164 fibrosarcoma cells

WEHI-164 cells death was calculated as Suppression Index (SuI) as: Absorbance at 540 nm of Test /absorbance at 540 nm of Control. Increase of Supernatant TNF-α results in decreased amount of SuI. All concentrations of 1, 10, 100 and 200 µg/ml of β-Glucan significantly (P = 0.023) increased the ability of macrophage supernatants to kill the cancer cell line

fungal cell wall are polysaccharides and glycoproteins. β-Glucan has been purified from brewer's and backer's yeast (Tokunakak *et al.*, 2000), from oats and barley bran (Baursk and Geisler., 1996). The healing and immunostimulatory properties of β-Glucan have been suggested for years. β-Glucan may be a powerful immune stimulant and be a beneficial antagonist to both benign and malignant tumors (Chang *et al.*, 2009). Glucans are thought to mediate their effects via interaction with membrane receptors on macrophages, neutrophils and NK cells. Macrophages play a critical role in all phases of host defense that are both innate and adaptive immune responses in case of an infection. macrophage can produce cytokine and inflammatory mediators such as nitric oxide, NO, and hydrogen peroxide, H_2O_2. Thus activation of macrophage functions by β-Glucans increases host immune responses. Some previous studies evaluated the effects of β-Glucan on macrophages in vitro and in the present study, we evaluated in vitro effects of β-Glucan on macrophage functions as NO release as a marker of inflammatory responses and anti-cancer potential of macrophage cells as a major component of the anti tumor immune responses. Our results showed that β-Glucan in all examined concentrations had no cytotoxic effect on macrophages and imply probable use of β-Glucan without toxic effects on the cells. β-Glucan at dose of 10 μg/ml significantly increased the NO production by peritoneal macrophages. A number of studies have indicated that polysaccharides stimulate NO but not in reactive oxygen intermediate production in a dose-dependent manner from peritoneum macrophages (Ohno *et al.*, 1996). Therefore, β-Glucan has a direct anti-cancer effect (Chang *et al.*, 2009) and can inhibit tumor development as well as reducing its proliferation. (Akramiene *et al.*, 2007). In order to evaluate the anti-tumor properties of Glucan, macrophage culture supernatants were affected on WEHI-164 fibrosarcoma cells. WEHI-164 cells are susceptible to the release of TNF-α from macrophages (Arora *et al.*, 2005). The results indicated that in all examined concentrations, tumoricidal TNF-α bioactivity which was compatible with IFN-γ positive control was enhanced. Increased cytotoxicity of macrophage supernatant culture on WEHI fibrosarcoma cell line suggests that β-Glucan could elevate the tumoricidal activity of macrophages as one of its indirect anti tumor activities. Moreover, fungal cell wall component has been found to be a major element in the activation of macrophages and the production of inflammatory mediators from these cells. (Chan *et al.*, 2009; Fernandesda *et al.*, 2010). These results indicate that β-Glucan could enhance proinflammatory agents production from macrophages and also increase the tumoricidal activity of macrophages on tumor cell line. These results are coordinate with other investigations

which have disclosed inhibitory effect of β-Glucan on tumor growth. In addition, the findings proved that β-Glucan increased the number of peritoneal macrophages receptors in molecular host-mediated mechanisms (Ukaway *et al.*, 2000). Natural components regulate macrophages functions. In particular, these compounds have been shown to increase macrophage cytotoxic activity against tumor cells and microorganisms, activate phagocytic activity, increase reactive oxygen species (ROS) and nitric oxide (NO) production and to enhance the secretion of cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1, IL-6, IL-8, IL-12, IFN-γ (Brummer *et al.*, 2007). The resulting antitumor effect of β-Glucan could be due to each of these mechanisms or a combination of several mechanisms. The discovery and identification of new and safe drugs without adverse side effects, is the ultimate goal of research in the biomedical science. Medicinal applications have been demonstrated for many traditionally used mushrooms, with large differences in immunomodulatory properties. The species studied so far represent a vast source of immunomodulating and anti tumor extracts and metabolite (Cristina *et al.*, 2005) . Finally, the results of the present study indicate that β-Glucan could enhance NO production from macrophages and increase the tumoricidal activity of macrophages on WEHI-164 fibrosarcoma cells. The results point to β-Glucan as a natural immunostimulatory agent with tumoricidal activity as well as its application in the treatment of cancer. Further studies are needed to clarify the advantages of this component in clinical applications.

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