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Immunomodulatory Activities Associated with β-Glucan Derived from *Saccharomyces cerevisiae*

A. C. PELIZON, R. KANENO, A. M. V. C. SOARES, D. A. MEIRA[1], A. SARTORI

Department of Microbiology and Immunology, Institute of Biosciences, and [1]Department of Tropical Diseases and Diagnosis by Image, Faculty of Medicine, State University of Sao Paulo, Sao Paulo, Brasil

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Summary

In this study we investigated the effect of β-glucan derived from *Saccharomyces cerevisiae* on fungicidal activity, cytokine production and natural killer activity. Spleen and peritoneal cells from female C57BL/6 mice, previously injected (24 or 48 h) with 20 or 100 μg of glucan by i.p. route, were assayed. *In vivo* β-glucan administration primed spleen cells for a higher production of IL-12 and TNF-α when *S. aureus* was used as a stimulus. In addition, β-glucan increased NK spleen cells activity against YAC target cells. Some immunomodulatory activities not yet described for β-glucan were observed in this work.

Key words

β-glucan • IL-12 • TNF-α • NK activity • Fungicidal activity

Introduction

Many polysaccharides activate cellular components involved in host defense mechanisms (Tzianabos 2000). β-glucan is derived from the yeast cell walls of species such as *Saccharomyces cerevisiae* and modulates many *in vivo* and *in vitro* activities (Estrada *et al.* 1997). Its main immunopharmacological activities are associated with anti-tumor effect (Di Luzio *et al.* 1979, Lotzová and Gutterman 1979), radioprotective action (Hofer and Pospíšil 1997), increased host resistance to viral, bacterial and parasitic infections (Di Luzio and Williams 1978, 1984, Browder *et al.* 1984, Sandula *et al.* 1999, Lee *et al.* 2001) and also adjuvant effects (Chirigos *et al.* 1978, Holbrook *et al.* 1981, Benach *et al.* 1982).

The exact β-glucan action mechanism is still unclear. Some experiments have shown that it does not directly activate leukocyte functions, such as oxidative burst (Mackin *et al.* 1994) or cytokine secretion (Poutsiaka *et al.* 1993). Although a few investigations have reported down-modulatory activities mediated by β-glucan (Masihi *et al.* 1997, Soltys and Quinn 1999), most of available findings point to positive modulatory activity. Glucan particles induced human monocytes to produce tumor necrosis factor-α (TNF-α) and interleukin-1β by a mechanism dependent on trypsin-sensitive β-glucan receptors (Abel and Czop 1992). Zymosan also induced interleukin-8 production by interaction with a CD11b/CD18 receptor on human neutrophils (Au *et al.* 1994). More recently, vitronectin and fibronectin have been used as glucan binding proteins increasing macrophage cytokine release (Olson *et al.*

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

1996, Vassallo *et al.* 2001). A different view of glucan biological activity is based on its priming or synergistic effects. Větvička *et al.* (1996) showed that soluble CR3-specific polysaccharides such as β-glucan induce a primed state of CR3 that can trigger the killing of iC3b-target cells that were otherwise resistant to cytotoxicity. Also, soluble β-1,3-glucan triggered a strong synergistic effect on lipopolysaccharide (LPS)-induced secretion of IL-8 and IL-10 and monocyte tissue factor activity. This glucan preparation also strongly primed for TNF-α and IL-6 induction by LPS (Engstad *et al.* 2002).

Paracoccidioidomycosis (PCM) is an endemic disease in most Latin American countries (Wanke and Londero 1994). A previous experience with PCM patients showed that intravenous administration of particulate β-glucan was clearly favorable to disease control (Meira *et al.* 1996). The present study was undertaken to evaluate if β-glucan modulates immunological mechanisms involved in the defense against *P. brasiliensis*. Cells from mice previously injected with β-glucan were tested for fungicidal activity, cytokine production and natural killer (NK) activity.

Methods

Animals

Four to six-week-old female C57BL/6 mice were used. Animals were maintained at the animal facility of the Department of Microbiology and Immunology, São Paulo State University (UNESP), Botucatu, Brazil, supplied with filtered water and conventionally fed (Nuvital – CR1) *ad libitum*. Room temperature was set at 22 ºC and lighting consisted of alternate 12 h light / dark cycles.

β-glucan preparation and treatment

Particulate β-glucan was prepared from *S. cerevisiae* at the Hemocenter of Botucatu School of Medicine (São Paulo State University) using the Hassid *et al.* (1941) method modified by Di Luzio *et al.* (1979), which is described below. Initially 1.5 kg of dry yeast was suspended in 2 liters of a 3 % aqueous sodium hydroxide solution. The suspension was placed in a boiling water bath for 4 h cooled overnight, and the supernatant decanted. This procedure was repeated three times. The residue was then acidified with 800 ml concentrated hydrochloric acid (HCl) plus two liters of 3 % HCl and placed in a boiling water bath for 4 h. The suspension was allowed to stand overnight and supernatant decanted. The residue was further digested with three liters of 3 % HCl at 100 ºC for 4 h, cooled overnight and decanted. This HCl digestion was repeated twice. The residue was then washed with distilled water three times at 20 ºC, and twice at 100 ºC. Then one liter of ethyl alcohol was added to the residue, mixed thoroughly, and allowed to stand at least 24 h for maximum extraction. The particulate β-glucan preparation was then collected by filtration and dispersed with a graal homogenizer. Flasks containing 50 mg of glucan were sterilized by autoclaving and a suspension was prepared for use by adding 5 ml of sterile endotoxin-free saline. To test if this preparation was contaminated with mannoproteins as described for a mannoprotein-β-glucan complex released from *C. albicans* (Kurihara *et al.* 2003), it was mixed with concanavalin A conjugated with FITC (Sigma). This reagent gave positive results with both *Candida sp*. and *S. cerevisiae* whole yeast cells, but no fluorescence was observed when β-glucan particles were assayed by the same protocol (not shown), suggesting that our β-glucan preparation was not contaminated with mannoproteins.

Mice (4 animals/group) were treated with glucan (20 or 100 μg/animal) 24 or 48 h before the sacrifice for peritoneal and splenic cell harvesting.

Fungi

P. brasiliensis strain 18 (Pb18) was maintained in yeast form at 35 ºC in GPY culture medium for six days. Yeast viability was determined by phase contrast microscopy and bright yeast cells were counted as viable, while dark ones were considered non-viable. Fungal suspensions containing more than 90 % viable cells were used for the experiments.

Fungicidal activity

Fungicidal activity associated with peritoneal macrophages was tested as previously described (Brummer *et al.* 1989). In brief, macrophage monolayers from mice treated or not with glucan, were infected with *P. brasiliensis*. After coculture during 4 h, macrophages were lysed with distilled water. The number of colony forming units (CFU) was determined by plating the suspension in brain-heart infusion agar medium containing normal horse serum and conditioned medium from *P. brasiliensis* strain 192.

Cytokine detection in culture supernatants

Spleen cells ($4x10^6$ cells/ml) from normal or

glucan-treated mice were cultured in 48-well tissue culture plates (Costar, Cambridge, MA) and stimulated with 0.004 % of fixed *S. aureus* (Pansorbin, Calbiochem – Behring, Co., La Jolla) in a humidified 5 % CO_2 incubator at 37 ºC. After 24 h, the supernatants were collected, stored at –20 ºC and later assayed for cytokine concentration by using a sandwich enzyme-linked immunosorbent assay. C15.6, 9A5 and IP-400 were used as capture antibodies for p40, p70 and TNF-α, respectively. Biotinylated anti-IL-12 (C17.8) and anti-TNF-α (MP6-XT3) were used as revealing antibodies. Standard curves were prepared with the recombinant cytokines.

Chromium release assay for NK cytotoxicity

NK activity was tested as previously described (Kaneno *et al.* 2004). In brief, NK activity of effector cells was measured by 4 h ^{51}Cr-release assay using labelled YAC-1 target cells (effector to target cells ratio was 50:1). Plates were incubated for 4 h at 37 ºC in a humidified CO_2 incubator. After incubation, plates were centrifuged at 1200 rpm for 10 min and 100 µl of supernatants were collected and radioactivity was measured using a Beckman Biogamma Counting System (Irvine, Calif.). Spontaneous release was determined by adding 100 µl labelled target cells to 100 µl of medium in the absence of effector cells. Percentage cytotoxicity, as measured by specific ^{51}Cr-release, was calculated by using the formula: 100 x (experimental cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm).

Statistical analysis

NK activity and cytokine production were expressed as the median values and statistically analyzed by a non-parametric ANOVA Test (Kruskall-Wallis). Fungicidal activity was expressed as the mean ± S.E.M. and analyzed by the one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison test. Differences were considered significant if p value was less than 0.05.

Results

β-glucan enhances fungicidal activity against P. brasiliensis

Peritoneal macrophages, obtained from C57BL/6 mice previously treated with β-glucan, showed a higher microbicidal activity against *P. brasiliensis*, in comparison with cells obtained from non- treated animals as detected by a decrease in viable fungi recovery. Although this effect was also observed with the higher dose, only the 20 µg/animal triggered a significant effect (Fig. 1).

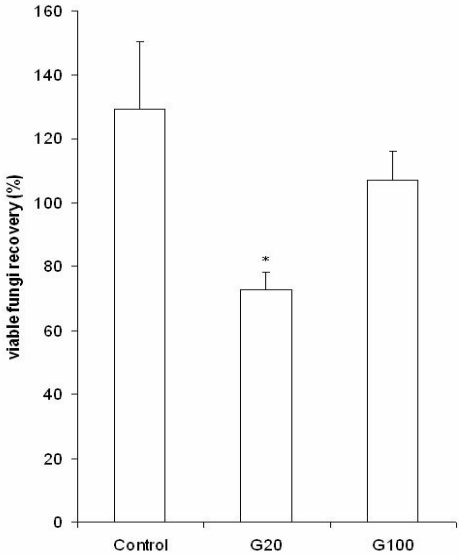

Fig. 1. β-glucan effect on fungicidal activity against *P. brasiliensis*. C57BL/6 mice were treated with β-glucan (20 µg/ml – G20 or 100 µg/ml – G100) by i.p. route. Fourty-eight hours later macrophages were collected from the peritoneal cavity and assayed for their fungicidal activity. The results are the mean ± SE for groups of 4 animals each. This is one of four similar experiments. * Significant difference between the group treated with 20 µg of glucan and the control.

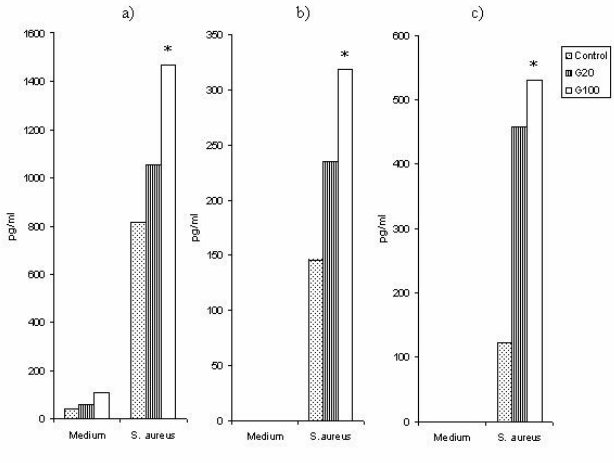

Fig. 2. β-glucan effect on IL-12p40 (**a**), IL-12p70 (**b**) and TNF-α (**c**) production. C57BL/6 mice were treated with β-glucan (20 µg/ml – G20 or 100 µg/ml – G100) by i.p. route. Spleen cells were obtained 24 h after glucan injection. Cell cultures were stimulated with *S. aureus* (0.004 %) and cytokines were quantified in 48 h supernatants by ELISA. The results are the median for groups of 4 animals each. This is one of three similar experiments. *Significant difference between the group treated with 100 µg of glucan and the control.

β-glucan primes for higher IL-12 and TNF-α production

As can be observed in Figure 2, splenic cells from mice previously treated with glucan, produced higher levels of IL-12p40 (2a), IL-12p70 (2b) and TNF-α (2c) when stimulated *in vitro* with *S. aureus*. This priming effect was significant with the dose of 100 μg.

β-glucan does not stimulate cytokine production in vitro

Splenic cells from normal mice were stimulated *in vitro* with a range of glucan concentrations. No cytokines (TNF-α, IL-12p40 and IFN-γ) were detected in the supernatants (results not shown).

β-glucan increases NK activity

The effect of pretreatment with glucan on NK activity is shown in Figure 3. Both glucan concentrations triggered an increase on NK activity, but the effect was significant only at the dose of 20 μg/animal.

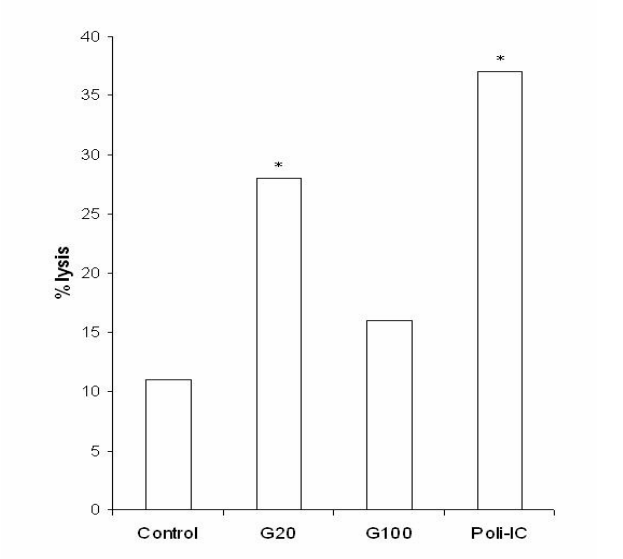

Fig. 3. β-glucan effect on splenic natural killer (NK) activity. C57BL/6 mice were treated with β-glucan (20 μg/ml – G20 or 100 μg/ml – G100) by i.p. route. Forty-eight hours later, non-adherent splenic cells were tested for their NK activity against YAC cells. The results are the median for groups of 4 animals each. This is one of four similar experiments. * Significant difference in comparison to control group.

Discussion

Previous work has shown that intravenous administration of β-glucan benefits PCM patients treated with anti-fungal drugs. Patients who received glucan, in spite of being more seriously ill, had a stronger and more favorable response to therapy than patients with the less serious disease treated only with anti-fungal. By analyzing different immunological parameters, it was concluded that glucan treatment clearly improved immune response. In addition, no side effects were noticed after glucan intravenous treatment (Meira *et al.* 1996). The present study was undertaken to evaluate if β-glucan modulates immunological mechanisms involved in *P. brasiliensis* elimination. To investigate this, β-glucan used in PCM patients treatment was tested *in vivo* and *in vitro* assays. The main protocol consisted in a previous β-glucan injection in C57BL/6 mice by i.p route followed by an *ex vivo* evaluation of fungicidal activity (peritoneal cells), cytokine production and NK activity (splenic cells). Peritoneal macrophages from mice injected with glucan (48 h before) showed a significantly higher ability to kill the fungus in comparison with cells from normal mice. This effect depended on the glucan dose, the dose of 20 μg/mouse being the most efficient. In the group treated with 100 μg/mice, we also observed a slight increase in the fungicidal activity which was not significantly different compared to the control group. In agreement with these results, it was recently described that the polysaccharide fraction of *P. brasiliensis* mycelial cell wall (F1 fraction), the component from which β-glucan derives, contributes to human monocytes for effective *P. brasiliensis* killing (Anjos *et al.* 2002). This finding is the first experimental demonstration of an increased fungicidal activity against *P. brasiliensis* promoted by purified β-glucan. This immunopotentiating effect could be, at least, one of the glucan activities that resulted in a stronger and more favorable response of PCM patients to anti-fungal therapy associated with glucan administration.

It is well established that IFN-γ and TNF-α are pivotal cytokines in macrophage activation necessary for destroying different infectious agents (Leenen *et al.* 1994). The contribution of these cytokines to *P. brasiliensis* killing was recently observed (Souto *et al.* 2000, Calvi *et al.* 2003). No TNF-α, IL-12 or IFN-γ were induced directly by *in vitro* addition of glucan to splenic cell cultures (data not shown). The direct induction of cytokines by glucan preparation is a controversial issue intensified by the multiplicity of glucan sources and methodologies used to obtain it (Brown and Gordon 2003). This lack of direct cytokine induction is in agreement with other reports (Poutsiaka *et al.* 1993, Adams *et al.* 1997, Wakshull *et al.* 1999). Interestingly, we observed that previous *in vivo* glucan administration to mice primed their cells to produce more TNF-α and

IL-12 in response to further *in vitro* stimulation with *S. aureus*. This priming effect for TNF-α production was previously described for other glucan preparations. Ohno *et al.* (1995) observed that administration of grifolan (100-250 μg/mouse) obtained from *Grifola frondosa*, did not elevate TNF-α concentration in serum. However, it significantly elevated LPS-elicited TNF-α production in serum. These effects were clearly related to the degree of glucan branching and molecular weight. The mechanism involved in this priming effect was not investigated by us, but it has been described by other authors (Adams *et al.* 1997, Battle *et al.* 1998), and could be mediated, at least in part, by activation of nuclear transcription factors, including NFκB and NF-IL-6.

In view of the fundamental role of IL-12 in the immune response, we considered the results related to this cytokine of a major importance. The possible contribution of IL-12 to glucan efficacy could be attributed to two different aspects. First of all this cytokine plays a major role on Th1 differentiation (Trinchieri 1995) that is ultimately responsible for the immune control of intracellular pathogens survival (Gately *et al.* 1998). In addition, this cytokine was originally described as an NK activating factor (Kobayashi *et al.* 1989). In our model, this activating effect was suggested by the finding that previous treatment with glucan triggered enhanced NK activity in the spleen. This increment was statistically significant in the group treated with the lower β-glucan dose (20 μg). Since the *in vitro* addition of β-glucan did not increase NK activity (results not shown) we believe that most of this increment could be associated with the induction of IL-12 production.

The importance of β-glucan priming effect for IL-12 production in the control of PCM is still unknown. However, some reports suggest that an escape mechanism involving IL-12 is probably hindering the development of an effective immune response against this fungus. IL-12 treatment of mice infected with *P. brasiliensis*, markedly inhibited yeast dissemination to liver and spleen (Calich *et al.* 1998). Recent corroborating evidence demonstrated that PCM patients show a defective IL-12 synthesis. Also, the addition of IL-12 or anti-IL-10 neutralizing antibody to their mononuclear cell cultures restored IFN-γ production (Romano *et al.* 2002). Reviewing the pathobiology of *P. brasiliensis*, Borges-Walmsley *et al.* (2002), described that transition from the environmental conidia to the pathogenic yeast form is accompanied by modulation of the cell wall composition. Notably, the polymer linkages change from β-glucan to α-glucan, possibly to avoid triggering of the inflammatory response by β-glucan. In this context, it is possible to imagine that this evasion mechanism includes prevention of IL-12 production. Consequently, β-glucan association with conventional anti-fungal treatment could be considered a potentially valuable therapeutic strategy. To adopt or support this kind of combined therapy it would be necessary to unravel the contribution of glucan (α and β) that are presumably free in paracoccidioidomycosis patients serum. Only one paper (Itano *et al.* 2002) demonstrated the presence of β-glucan in some plasma samples from paracoccidioidomycosis patients. On the other hand, no description of soluble and free α-glucan in patient´s serum is available. Several reports show a clear change from β-glucan to α-glucan during transition from mycelia to yeast form in pathogenic fungi (Klimpel and Goldman 1988, Hogan and Klein 1994, Borges-Walmsley *et al.* 2002). This transformation is possibly avoiding the β-glucan triggering of the immune response and therefore, facilitating fungi infection. Based on this, it is tempting to speculate that α-glucan is preferentially present in patient´s serum and that this could favor fungi spread. In this context, β-glucan administration, by virtue of its immunostimulatory potential, would be promising in paracoccidioidomycosis treatment.

Interestingly, but hard to explain, was the relationship between the doses and the biological modifications. The lower dose was more effective to increase NK and fungicidal activity. On the other hand, the higher dose was more effective in priming cells for higher cytokine production. The mechanism underlying these differences was not investigated by us, but a dose-dependence was also reported by other authors (Lotzová and Gutterman 1979, Lee *et al.* 2001). In this context, we could imagine that priming for cytokine production could require a stronger signal, and therefore, depends on a higher glucan concentration. Another possibility is the presence of a selective toxic effect, caused by a higher glucan concentration, on NK and fungicidal activities.

Together, our results suggest that β-glucan derived from *S. cerevisiae* is able to improve immune functions that contribute to *P. brasiliensis* elimination.

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Reprint requests

A.C. Pelizon, Department of Microbiology and Immunology, Instituto de Biociências, Universidade Estadual Paulista (UNESP), SP, Brasil, 18618-000. E-mail: sartori@ibb.unesp.br