

Comparison of Immunological Effects of Commercially Available β -Glucans: Part III

Abstract

β -Glucans represent the most studied natural immunomodulators. With the well-described structure and function, the use of glucans slowly but steadily progresses from supplements to drug. However, direct comparisons of biological activities of individual glucans are rare. As this study will show, no direct connection between source and immunological activities was found. Based on these results, we can conclude that highly purified and highly active glucans have strong and pleotropic effects, whereas poorly defined glucans have only medium (if any) biological effects.

Research Article

Volume 2 Issue 4 - 2016

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Received: May 31, 2016 | Published: June 28, 2016

Introduction

β -D-glucans (referred to further on in this text as “glucans”) form a part of a group of physiologically active compounds called “biological response modifiers” and represent highly conserved structural components of cell walls in yeast, mushroom, and seaweed. Generally, glucan (sometimes β -glucan) is the chemical name of a polymer of β -glucose. In past decades, natural glucans were sometimes considered to be “biological immunomodulators,” or “biological response modifiers,” and sometimes as “pathogen-associated molecular patterns.” None of these terms are accurate, since they usually focus on only a few effects. Polysaccharides in general and glucans in particular, have a long history as immunomodulators. As early as the beginning of the 18th century, it was known that certain infectious diseases showed a therapeutic effect on malignant processes. The Documented history of polysaccharides as immunomodulators goes back to the 1940s when Shear and co-workers [1] described a substance from *Serratia marcescens* cultures that caused tumor necrosis. During decades of research, numerous types of glucan have been isolated and described.

In scientific literature, you can find hundreds of different components, all under the name glucan. With over 9,000 published studies on the biological effects of glucan, it is clearly the most studied immunomodulator (for review see [2]). Unfortunately, not all glucans were created equal and glucans widely differ not only in physicochemical properties such as branching or molecular weight, but also in biological properties. Some of the described glucans show little activity and some have no biological activities. It is necessary to constantly monitor all conditions during the isolation and purification processes; otherwise the final product will have limited biological activity, if any. The concentration of effective glucan in a product causes a strong relationship to immunological effects. The considerable heterogeneity of all natural glucans, not only from *Saccharomycetes* but also from

other sources, obviously was and continues to be the cause of a series of mutually contradicting conclusions. An excellent review of glucans as biological response modifiers and the relationship between structure and functional activity is given in Bohn & BeMiller [3]. Despite extensive investigations, no consensus on the source, size, or other properties of glucan has been reached. An important comparison of yeast-derived and mushroom-derived glucans and their biological activities is given in Kogan [4], Vetvicka & Vetvickova [5-7]. With so many reports showing the significant effects of glucan on various biological (and most of all immunological) activities, one would assume that after 40 years of extensive research, glucan would already be widely accepted immunostimulants. However, some problems remain and they substantially lower enthusiasm of regulating agencies.

One of the problems is the fact that, despite the overwhelming number of scientific reports, far too many individual glucans have been used that differ widely in source, solubility, molecular weight, branching and other characteristics. In addition, various concentrations and routes of administration (oral, intraperitoneal, intravenous, subcutaneous) have been tested. All this leads to confusion, with numerous manufacturers claiming that their glucan possesses the highest biological activities. The problem of diverse data can be solved only by comparative studies. However, scientific reports directly comparing individual glucans are limited [5,8-11]. This led us to the current comparative review of 16 different commercially available glucans. This study represents a part III of direct glucan comparisons [7,12].

Material and Methods

Animals

Female, 8 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by cervical dislocation.

Material

All glucans were either donated or purchased from the manufacturers as shown in Table 1. Lipopolysaccharide (LPS) and cyclophosphamide were purchased from Sigma (St. Louis, MO, USA).

Cell lines

Human myeloblastic cell line HL-60 and human lung cancer cell line NCI-H23 were obtained from the ATCC (Manassas, VA). The Lewis lung carcinoma cells were obtained from Dr. G. Ross (University of Louisville, Louisville, KY) and were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) medium containing HEPES (Sigma) buffer supplemented with 10% heat-inactivated FCS (Hyclone Lab., Logan, UT), without antibiotics, in plastic disposable tissue culture flasks at 37 °C in a 5% CO₂/95% air incubator.

Phagocytosis

Phagocytosis of synthetic polymeric microspheres was described earlier [7].

Nitrite production

For nitrite (NO₂⁻) formation we employed a technique described in Green & Nacy [13] with LPS as triggering agent.

IFN γ production

Twenty four hours after ip. Injection with 100 μ g of individual samples suspended in PBS, the mice were sacrificed, blood collected, serum prepared and filtered through 0.45 μ m filter. The level of IFN γ was determined using Quantikine mouse IFN γ kit (R & D Systems, Minneapolis, MN, USA) as described earlier [10].

IL-2 secretion

Purified spleen cells (2x10⁶/ml in RPMI 1640 medium with 5% FCS) obtained from mice injected with 100 μ g of individual sample or PBS was added into wells of a 24-well tissue culture plate. Cells were incubated for 48h in a humidified incubator (37 °C, 5% CO₂/95% air). Addition of 1 μ g of Concanavalin A (Sigma) was used as a positive control. At the endpoint of incubation, supernatants were collected, filtered through 0.45 μ m filters and tested for the presence of IL-2 using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

Lewis lung carcinoma therapy

Mice were injected i.m. with 5x10⁶ of Lewis lung carcinoma cells. Cyclophosphamide (150 mg/kg) was used i.p. at day 10 after tumor application. Individual samples were used i.p. (200 μ g/mouse) from day 0 to day 14 after tumor application. The control group of mice received daily i.p. PBS. Each group held a minimum of 5 mice. At the conclusion of the experiment, mice were euthanized, lungs removed, fixed in 10% formalin and the number of hemotogenic metastases in lung tissue was estimated using a binocular lens at 8x magnification.

Statistics

Student's t-test was used to statistically analyze the data. Data at p<0.05 were considered significantly different.

Results

Nobody really knows how many glucans are commercially available throughout the world. Not only can we use numerous sources (such as yeast, fungi, seaweed or grains), but the results will differ based on the isolation used. In our ongoing search for the best commercial glucan, we evaluated 14 new commercially available glucans from several countries and compared them with Glucan #300, which was previously shown to have superior effects. Individual glucans and their manufacturers are given in Table 1. The effects of glucan on cellular immunity are well established. Phagocytosis is, therefore, the test of choice for evaluation of glucans activities, as it is very rare that glucan not affecting Phagocytosis would have additional immunostimulating effects. We used the synthetic polymeric microbeads known for their minimal spontaneous adhesion to the cell membrane, thus eliminating false positivity [14]. Our results are summarized in Table 2 and show that some glucans are not active even in massive 800 μ g dose, whereas other glucans (such as Reishi Mushroom Extract, Beta 1,3/1,6-D-Glucan, Yestimun, or Beta Glucan) showed clear dose-dependency. In general, Glucan #300 was again the most active glucan showing significant effects even at the lowest 25 μ g dose.

Phagocytosis, which originally was the main means of cell feeding, is in fact a simple internalization of material. This biological activity is usually followed up with a burst of metabolic activity and production of a series of biologically active oxygen species. In our study we focused on nitrite oxide production. From data shown in Table 3 we can see that almost all tested glucans (with exception of Beta Glucan, and barley beta Glucan) significantly stimulate nitride oxide production. Among the most active glucans were Glucan #300, Yestimune and Beta Glucan. With strong effects on cellular immunity, it is not surprising that glucan affects the synthesis and release of several cytokines. In our study, we evaluated the effects of tested glucans on the production of IFN- γ in the blood and IL-2 by splenocytes (*in vitro*). Table 4 shows the glucan-mediated production of IFN- γ . As the unstimulated mice showed almost no IFN- γ (2.2pg/ml), it is not surprising that all glucans caused statistically significant increase in IFN- γ secretion. The most active samples were Glucan #300 and Yestimune. Similar results were obtained when we evaluated the effects on glucan-induced IL-2 production. Again, the unstimulated splenocytes produced no IL-2, therefore all glucans stimulated significantly higher production. The most active samples were Glucan #300, Yestimun and Reishi Mushroom Extract (Table 5).

The last part of our study was devoted to the effects on inhibition of cancer. Using a well-defined Lewis lung carcinoma cell model, we found that only Glucan #300, Yestimun, Beta Glucan and Beta 1,3/1,6-D-Glucan had significant effects in cancer reduction. In all other cases, the effects were either not statistically significant or there were not effects at all.

Table 1: Types of glucan used.

Glucan	Source	Manufacturer
Beta Glucan	Oat	Bioimersion, Bellevue, WA, USA
Organic Immuno-build Mushrooms	Mushroom	Rainbow Light Nutritional Systems Santa Cruz, CA, USA
Reishi Mushroom Extract	Mushroom	Mehdi Reishi
Beta Glucan	Yeast	Cape Fear Naturals, Wilmington, NC, USA
Beta 1,3 Glucans	Yeast	The Vitamin Shoppe, North Bergen, NJ, USA
Beta 1,3/1,6-D-Glucan	Yeast	Piping Rock, Ronkonkoma, NY, USA
β -Glucan Ball	Mushroom	Umeken, Cerritos, CA, USA
Beta Glucan	Yeast	Vistra, Thailand
Barley Beta Glucan	Barley	Doctor's Best, Irvine, CA, USA
Beta Glucan	Mushroom	Nef De Sante, Prague, Czech Republic
Yestimun	Yeast	Leiber, Bramsche, Germany
Sangraksu Chaga Mushroom	Mushroom	Betaglucan Korea, Seoul, Korea
Beta 1,3 Glucan	Mushroom	Douglas Laboratories, Pittsburg, PA, USA
Beta Glucan	Yeast	Source Naturals, Santa Cruz, CA, USA
#300	Yeast	Transfer Point, Columbia, SC, USA

Table 2: Effects of dose on Phagocytosis.

Dose (mg/ml)	25	50	100	200	400	800
Beta Glucan	28.8 ± 2.7	30.0 ± 2.7	31.6 ± 3.1	31.6 ± 2.6	33.8 ± 3.9	34.8 ± 2.1
Organic Immuno-build Mushrooms	30.4 ± 2.2	30.9 ± 3.8	31.6 ± 3.4	34.7 ± 4.0	36.5 ± 3.5	37.1 ± 2.7*
Reishi Mushroom Extract	30.5 ± 2.6	33.4 ± 4.1	35.6 ± 2.8	37.8 ± 2.9	40.1 ± 2.8*	40.5 ± 3.8*
Beta Glucan	28.9 ± 2.5	30.0 ± 2.4	32.7 ± 3.1	34.1 ± 4.1	33.8 ± 3.8	35.2 ± 4.1
Beta 1,3 Glucans	30.1 ± 2.7	32.1 ± 1.9	33.0 ± 2.8	33.8 ± 2.2	35.4 ± 2.9	35.1 ± 3.3
Beta 1,3/1,6-D-Glucan	34.0 ± 2.5	36.8 ± 2.9	37.9 ± 2.8*	40.1 ± 3.4*	41.1 ± 3.5*	42.0 ± 3.7*
β -Glucan Ball	28.8 ± 2.1	33.1 ± 2.5	30.8 ± 1.7	32.4 ± 2.2	33.9 ± 2.1	36.5 ± 2.8
Beta Glucan	29.9 ± 3.2	33.1 ± 3.1	34.5 ± 1.9	33.2 ± 3.1	34.1 ± 3.0	38.1 ± 4.1*
Barley Beta Glucan	30.1 ± 2.8	31.3 ± 2.8	32.1 ± 4.1	32.9 ± 1.9	35.1 ± 4.1	33.5 ± 2.5
Beta Glucan	30.8 ± 2.2	32.8 ± 2.7	32.1 ± 1.9	36.3 ± 2.3	34.9 ± 2.1	39.6 ± 3.1*
Yestimun	33.8 ± 1.8	44.5 ± 2.7*	46.6 ± 3.2*	47.9 ± 3.1*	48.8 ± 2.0*	49.9 ± 3.3*
Sangraksu Chaga Mushroom	30.4 ± 3.4	33.8 ± 2.2	43.8 ± 1.1*	44.5 ± 2.7*	45.8 ± 2.1*	43.5 ± 4.5*
Beta 1,3 Glucan	31.1 ± 2.2	32.9 ± 1.9	35.2 ± 3.8	36.3 ± 2.7	38.1 ± 2.9*	37.6 ± 2.5*
Beta Glucan	29.5 ± 1.8	33.1 ± 1.9	37.8 ± 3.0*	40.1 ± 2.5*	42.2 ± 2.9*	44.4 ± 4.1*
#300	42.3 ± 2.1*	47.8 ± 2.0*	54.8 ± 3.2*	56.5 ± 3.2*	54.8 ± 3.1*	61.7 ± 3.5*

*Significant difference between tested groups and PBS control group at $P \leq 0.05$ level. Results represent mean values from three experiments ± SD. Control (PBS) levels were 30.5 ± 2.7.

Table 3: Effects of glucan on nitrite oxide production.

Beta Glucan	1.01 ± 0.34*
Organic Immuno-build Mushrooms	0.76 ± 0.33*
Reishi Mushroom Extract	1.67 ± 0.23*
Beta Glucan	1.11 ± 0.42*
Beta 1,3 Glucans	1.02 ± 0.26*
Beta 1,3/1,6-D-Glucan	2.64 ± 0.11*
β -Glucan Ball	0.78 ± 0.35*
Beta Glucan	0.12 ± 0.38
Barley Beta Glucan	0.34 ± 0.22
Beta Glukan	1.06 ± 0.24*
Yestimun	3.89 ± 0.45*
Sangraksu Chaga Mushroom	1.01 ± 0.26*
Beta 1,3 Glucan	0.45 ± 0.11*
Beta Glucan	2.78 ± 0.33*
#300	6.34 ± 1.65*
PBS	0.08 ± 0.02

*Significant difference between tested groups and PBS control group at $P \leq 0.05$ level. Results represent mean values from three experiments ± SD.

Table 4: Effects of glucan on production of IFN- γ .

Beta Glucan	27.9 ± 3.3*
Organic Immuno-build Mushrooms	16.6 ± 2.2*
Reishi Mushroom Extract	33.0 ± 2.6*
Beta Glucan	4.9 ± 0.8*
Beta 1,3 Glucans	15.1 ± 1.1*
Beta 1,3/1,6-D-Glucan	37.6 ± 2.5*
β -Glucan Ball	13.2 ± 5.5*
Beta Glucan	34.8 ± 7.1*
Barley Beta Glucan	27.5 ± 4.4*
Beta Glukan	18.2 ± 2.1*
Yestimun	111.4 ± 7.9*
Sangraksu Chaga Mushroom	16.2 ± 2.3*
Beta 1,3 Glucan	15.5 ± 3.3*
Beta Glucan	66.2 ± 5.1*
#300	198.2 ± 8.9*
PBS	2.2 ± 0.1

*Significant difference between tested groups and PBS control group at $P \leq 0.05$ level. Results represent mean values from three experiments ± SD. Results are in pg/ml.

Table 5: Effects of glucan on secretion of IL-2.

Beta Glucan	226.5 \pm 38.6
Organic Immuno-build Mushrooms	76.8 \pm 21.1
Reishi Mushroom Extract	311.8 \pm 56.5
Beta Glucan	216.7 \pm 34.3
Beta 1,3 Glucans	111.1 \pm 25.7
Beta 1,3/1,6-D-Glucan	272.9 \pm 66.5
β -Glucan Ball	43.4 \pm 11.2
Beta Glucan	39.6 \pm 21.0
Barley Beta Glucan	101.3 \pm 52.1
Beta Glucan	277.5 \pm 66.9
Yestimun	543.8 \pm 87.1
Sangraksu Chaga Mushroom	116.0 \pm 32.7
Beta 1,3 Glucan	90.1 \pm 23.5
Beta Glucan	55.2 \pm 11.9
#300	828.7 \pm 101.5
PBS	0
Con A	1 067.3 \pm 299.2

All difference between tested groups and PBS control group are significant at $P \leq 0.05$ level. Results represent mean values from three experiments \pm SD.

Discussion

Glucans are natural immunomodulators, which due to numerous scientific studies and a significant amount of clinical trials have gained significant attention of not only scientists, but also the general public. With the approval as official drug in Japan in 1983 [15], glucan has a strong potential to be considered an official drug in Western medicine, too. In addition, numerous recent clinical trials confirmed the positive role of glucan supplementation in children with chronic respiratory problems [16] or in cancer patients [17]. However, as individual glucans differ from each other due to the differences in physicochemical properties and in biological activities, it is rather difficult to compare the effects described in the literature. The real comparison is possible only when individual glucans are directly compared in one study. However, these studies are relatively rare [11,4,18]. With the ever increasing amount of commercial glucans, we decided to run Part III of our comparative investigation [7,12].

In our two previous studies, we compared 30 different glucans differing in source (mushroom, yeast, barley and oat) and

solubility. The constant multiplication of commercially available glucans together with often questionable activities of some of the tested glucans led as to this Part III study. In the present paper, we used some of the same reactions, such as Phagocytosis, nitrite oxide formation, IL-2 and IFN- γ formation, and added a lung cancer model. Therefore, direct comparison with older studies is possible (Table 6).

For Phagocytosis, we used a 2-hydroxymethyl methacrylate microspheres model known for minimal spontaneous adhesion to cell surface, eliminating false positivity [14]. Our data showed that 30% of glucans had no significant activity even at the highest dose, but the best glucan showed strong activity even at the lowest dose. The idea that with less active glucans you can just increase the dose and get the same results is false, as glucans with low activity did not reach the stimulation of the best glucan even when used at 32x higher dose. Metabolic (respiratory) burst represents an important part of internalization of most materials. Respiratory burst plays an important role in the immune system and is a crucial reaction that occurs in phagocytes to degrade

internalized particles and bacteria. Sustained production of nitric oxide endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumor cells. The antimicrobial and cytotoxic actions of nitric oxide are enhanced by other macrophage products such as acid, glutathione, cysteine, hydrogen peroxide, or superoxide [19]. As several glucans have shown to stimulate oxidative burst [20], we measured the effects on nitrite formation. Our data clearly showed that most glucan had stimulating activity, with only two being not active in nitrite oxide stimulation. Several cytokines are known to be affected by glucan supplementation and these effects were confirmed in both animal and human models [21-23]. The only glucan without any effects on cytokines is Betafectin [24]. In

our study, we focused on production of IL-2 by splenocytes *in vitro* and on production of IFN- γ *in vivo*. Under normal steady-state conditions, splenocytes produce no IL-2, which is the reason why all glucan to have significant effects. However, only Glucan #300 showed activity close to that of Concanavalin A. The other glucans with strong activity on IL-2 production were Reishi Mushroom Extract and Yestimun. A rather similar situation has been found in case of IFN- γ , where the most active samples were again Glucan #300 and Yestimun. Glucan's effects on cancer growth are well established [15,25-27] therefore we tested our samples on mouse lung cancer model. Only four samples showed significant results leading to suppressed cancer growth – Glucan #300, Yestimun, Beta 1,3/1,6-D-glucan and Beta Glucan from Source Natural.

Table 6: Effects of glucan on suppression of lung cancer.

Beta Glucan	22.1 \pm 1.9
Organic Immuno-build Mushrooms	21.2 \pm 2.6
Reishi Mushroom Extract	18.2 \pm 3.6
Beta Glucan	19.5 \pm 2.7
Beta 1,3 Glucans	20.8 \pm 3.8
Beta 1,3/1,6-D-Glucan	16.9 \pm 3.4*
β -Glucan Ball	20.7 \pm 3.2
Beta Glucan	24.5 \pm 2.6
Barley Beta Glucan	23.6 \pm 2.5
Beta Glukan	21.1 \pm 2.9
Yestimun	15.3 \pm 1.7*
Sangraksu Chaga Mushroom	20.5 \pm 2.2
Beta 1,3 Glucan	22.1 \pm 1.9
Beta Glucan	16.3 \pm 3.5*
#300	11.7 \pm 1.2*
PBS	24.6 \pm 2.1

*Significant difference between tested groups and PBS control group at $P \leq 0.05$ level. Results represent mean values from three experiments \pm SD. Data represent number of lung metastases.

Conclusion

The third part of our ongoing investigation of commercially available glucans clearly demonstrated that several differences among samples exist, which might be an explanation for sometimes confusing results found in the literature. Similarly to our previous two comparisons [7,12], we tested 15 different glucans differing in source (mushroom, yeast, barley and oat). Again, Glucan #300 served as a benchmark. Our study confirmed that where there is

no basal level (IL-2 or IFN- γ), all or at least most glucans showed significant activity. However, in other biological activities, most of the glucans showed very limited if any activity, which was most clear in case of cancer growth. Clearly, individual glucans differ in biological effects based on tested characteristics. No clear relevance between the source used for isolation and biological effects has been found. From all samples, the Glucan #300 was the most active sample.

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