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.

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 therapeutical 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 Saccharomyces 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.

IFNγ production

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

IL-2 secretion

Purified spleen cells (2x10⁴/ml in RPMI 1640 medium with 5% FCS) obtained from mice injected with 100 µg 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 µg of Concanaulalin A (Sigma) was used as a positive control. At the endpoint of incubation, supernatants were collected, filtered through 0.45 µm 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 5x10⁴ 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 µg/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 6x 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 µg 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 µg 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 nitrite 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.
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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 Glukan             | 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 Glukan  | 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 ≤ 0.05 level. Results represent mean values from three experiments ± SD. Control (PBS) levels were 30.5 ± 2.7.

Citation: Vetvicka V, Vetvickova J (2016) Comparison of Immunological Effects of Commercially Available β-Glucans: Part III. Int Clin Pathol J 2(4): 00046. DOI: 10.15406/icpjl.2016.02.00046
Table 3: Effects of glucan on nitrite oxide production.

| Glucan Type                      | Mean ± SD  |
|----------------------------------|------------|
| 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 ≤ 0.05 level. Results represent mean values from three experiments ± SD.

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

| Glucan Type                      | Mean ± SD  |
|----------------------------------|------------|
| 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 ≤ 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.

| Glucan                          | IL-2 (pg/mL) ± SD |
|---------------------------------|-------------------|
| Beta Glucan                     | 226.5 ± 38.6      |
| Organic Immuno-build Mushrooms  | 76.8 ± 21.1       |
| Reishi Mushroom Extract         | 311.8 ± 56.5      |
| Beta Glucan                     | 216.7 ± 34.3      |
| Beta 1,3 Glucans                | 111.1 ± 25.7      |
| Beta 1,3/1,6-D-Glucan           | 272.9 ± 66.5      |
| β-Glucan Ball                   | 43.4 ± 11.2       |
| Beta Glucan                     | 39.6 ± 21.0       |
| Barley Beta Glucan              | 101.3 ± 52.1      |
| Beta Glukan                     | 277.5 ± 66.9      |
| Yestimun                        | 543.8 ± 87.1      |
| Sangraksu Chaga Mushroom        | 116.0 ± 32.7      |
| Beta 1,3 Glucan                 | 90.1 ± 23.5       |
| Beta Glucan                     | 55.2 ± 11.9       |
| #300                            | 828.7 ± 101.5     |
| PBS                             | 0                 |
| Con A                           | 1067.3 ± 299.2    |

All difference between tested groups and PBS control group are significant at P ≤ 0.05 level. Results represent mean values from three experiments ± 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.**

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

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