β1,3-Glucan: Silver Bullet or Hot Air?

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Abstract: β-Glucans belong to a group of biologically active natural compounds called biological response modifiers. These substances represent highly conserved structural components of cell walls in yeast, fungi, grain and seaweed. The effects of β-glucan on immune reactions are well established however direct comparisons of the biological activities of several individual glucans are extremely rare. As this paper will show, we tested sixteen different glucans and evaluated the possibility whether individual glucans will be similarly active against each of the tested biological properties or if each glucan will affect different reactions. No direct connection between source and immunological activities was found. Based on our results, we can conclude that highly purified and highly active glucans have pleotropic effects, whereas poorly isolated glucans have only average (if any) biological effect.

Keywords: Glucan; phagocytosis, IL-2, immunity, antibodies.

INTRODUCTION

β1,3-Glucan's role as an immunomodulator has been well documented for over 50 years. Initial interest in the immunomodulatory properties of polysaccharides was raised after experiments revealed that a crude yeast cell preparation stimulated macrophages *via* activation of complement [1]. Further work identified the immunomodulatory active component as β1,3-glucan [2]. β-Glucans show notable physiological effects; this is their most important quality and the reason why so much attention has been focused on them. Numerous studies (currently more than 6,000 publications) have subsequently shown that β1,3-glucans, either particulate or soluble, exhibit immunostimulating properties, including antibacterial and anti-tumor activities (for review see [3,4]).

There are various natural sources of β-glucans; however, they are most often prepared from fungal cell walls. Baker's yeast is the most common and likely the best raw material for glucan extraction. Additional sources of glucan involve mushrooms, grain and seaweed. Diverse data on comparison of structure, molecular size, and biological effects can be found in literature. For example, the antitumor activity of schizophyllan is supposedly conditioned by the triple helix presence and a molecular weight higher than 100 kDa. It is more than likely that the triple helix structure is not the sole effective form of β-glucan, because alkalic treatment, used in most isolation procedures, destroys this structure. In addition, the most recent opinions do not confirm the established ideas of the necessity of high molecular mass and branching of biologically active β-glucans.

Glucan is not only the most studied natural immunomodulator, it is also extremely succesfull commercially with numerous manufacturers/resellers active in every country. Because of the lack of comprehensive reviews comparing the biological effects of glucans isolated from various sources, and despite extensive investigations, no final conclusion has been reached. Therefore, there is not a single research paper willing to state that one source of glucan is better than another. In addition, numerous concentrations and routes of administration have been tested, including intraperitoneal, subcutaneous, and intravenous applications. This takes us back to the original question: which, out of dozens if not hundreds of individual glucans on the current market, is the best one? Which one has superior biological and/or immunological properties? For years, there was a controversy between the notions that water-insoluble glucans show only little biological activity, whereas soluble glucans are highly active. Similar questions whether orally-given glucan is as active as injected one has been solved only recently [5]. Based on limited published comparisons of individual glucans [5-8], we decided to compare numerous commercially available glucans.

MATERIALS AND METHODOLOGY

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 CO_2 asphyxiation.

Material

Individual glucans were purchased from the manufacturers or distributors as shown in Table **1**.

Cell Lines

Human neutrohil cell line HL-60 and human myeloid cell line U937 were obtained from the ATCC (Manassas, VA). The cell lines 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), 100U/ml penicillin (Sigma) and 100μg/ml streptomycin (Sigma), in plastic disposable tissue culture flasks at 37 °C in a 5% CO_2/95% air incubator.

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Table 1. Types of Glucan Used in this Study

Glucan	Source	Solubility	Manufacturer	Reference
Maitake Gold	Mushroom	Soluble	NutraGenesis, Brattleboro, VT	[6]
Krestin	Mushroom	Soluble	Kureha Corp, Japan	[5]
Aktival	Yeast	Insoluble	Farmal, Croatia	
Immutol	Yeast	Insoluble	Biotec ASA, Norway	[7]
Now Glucan	Yeast Mushroom	Insoluble Soluble	Now Foods, Bloomingdale,IL	[7]
Sweet Beta Glucan	Yeast	Soluble	K2 Global, Las Vegas, NV	
Beta Right 101	Yeast	Insoluble	Biothera, Eagan, MN	[26]
Epicor	Yeast	Insoluble	Vitamin Research Products, Carson City, NV	
NSC	Yeast	Insoluble	Nutritional Scientific Corp., Liberty, TX	[27]
Glucagel	Barley	Soluble	PolyCell Technologies, Crookston, MN	[7]
Better Immunity	Yeast	Insoluble	Young Again Nutrients, Magnolia, TX	
Macroforce	Yeast	Insoluble	ImmuneDyne, Houston, TX	[28]
Solgar	Yeast	Insoluble	Solgar, Leonia, NJ	
Wellmune	Yeast	Insoluble	Biothera, Eagan, MN	[26]
Swanson	Yeast	Insoluble	Swanson Health Products, Fargo, ND	
Glucan #300	Yeast	Insoluble	Transfer Point, Columbia, SC	[7]

Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier [9,10]. Briefly: peripheral blood cells or isolated peritoneal cells were incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml). The test tubes were incubated at 37° C for 60 min., with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive. Mice were injected with either glucan, glucan/resveratrol or PBS (control). All experiments were performed in triplicate. At least 200 cells in 60 high power fields were examined in each experiment.

IL-2 Production

Purified spleen cells ($2x10^6$/ml in RPMI 1640 medium with 5% FCS) from mice injected with glucan were added into wells of a 24-well tissue culture plate. After addition of 1 μg of Concanavalin A, cells were incubated for 48 hrs in a humidified incubator (37° C, 5% CO_2). 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).

Antibody Formation

Formation of antibodies was evaluated using ovalbumin as an antigen. Mice were injected twice (two weeks apart) with 100 μg of albumin and the serum was collected 7 days after last injection. Experimental groups were getting daily ip. injections of glucan. Level of specific antibodies against ovalbumin was detected by ELISA. As positive control, combination of ovalbumin and Freund's adjuvant was used.

Superoxide and Nitrite Production

Cells were incubated in a final volum of 200 ul of medium containing 0.1 % gelqatin and 100 uM cytochrome C (Sigma, St. Louis, MO, USA). Mice were challenged with 100 ug of individual glucans 24 hrs earlier. Cell lines were incubated with 1 ug/ml of glucans for 24 hrs. For the superoxide production, the reaction was initialized by addition of 5 ng/ml PMA (Sigma). After gentle mixing, the absorbance was measured 30 minutes after incubation at 37° C using multiwell spectrophotometer at 550 nm. Results are expressed as nanomoles of cytochrome C reduced/2.5 x 10^5 cells/30 minutes, after subtraction of the SOD and spontaneous release controls [11].

For a nitrite (NO_2^-) formation we used a technique described by Green and Nacy [12] with LPS (Sigma) as triggering agent.

RESULTS

The number of individual glucans is almost as great as the number of sources used for their isolation. The rationale for this combination of glucan samples was not only their commercial availability and success, but most importantly, our effort to include both soluble and insoluble glucans and also glucans isolated from different sources. Sixteen different glucans used for our comparison represented glucans available both on the U.S. and international markets. We

originally started with almost 50 different glucans, however for further testing we narrowed our choice to the sixteen ones. Information about individual types and manufacturer/distributors are given in Table **1**.

β-Glucans are generally considered to be potent stimulators of cellular immunity, with macrophages and neutrophils being the most important target. Not surprisingly, we started our evaluation of glucan activities by phagocytosis. We used the synthetic polymeric microspheres HEMA, since their use, dose and timing is already well established in glucan studies [13,14]. Results summarized in Table **2** show the effects of glucan samples on phagocytosis of synthetic particles by peripheral blood neutrophils. Our data suggested dose dependency, but only in sufficiently active glucans. Some glucans were either active only in extremely high doses (Sweet Beta Glucan) or showed only small and non-significant stimulation of phagocytosis (Epicor, Better Immunity, Macroforce, Solgar. Swanson). In general, only Glucan #300 stimulated the reaction even at the lowest dose.

Internalization of material is just one step in the complex process of phagocytosis. Another step is connected with a burst of metabolic activity and involves production of active oxygen species. In order to further evaluate the effects of tested glucans on phagocytosis, we decided to measure the production of two important molecules—superoxide anion and nitrite oxide. To be sure that we cover all possible effects, we used both fresh mouse neutrophils and two established cell lines— neutrophil cell line HL-60 and monocyte cell line U937. Data summarized in Table **3** showed that whereas almost all glucans stimulated superoxide anion production (with exception of Aktival), there were strong differences among individual samples with the most active glucans being Glucan #300, Wellmune, Maitake Gold, Krestin and Glucagel. Evaluation of nitrite oxide showed similar results (Table **4**). Surprisingly, in this case Aktival showed strong effects.

Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by spleen cells (Table **5**). The production of IL-2 was measured after a 48 hr *in vitro* incubation of spleen cells isolated from control and glucan-treated mice. Since the secretion of IL-2 by non-stimulated splenocytes (PBS) was always zero, every tested glucan showed significant stimulation of IL-2 production, with the most active glucans being Glucan #300, Wellmune, Maitake Gold, Beta Right and Krestin. None of the glucans, however, reached the effects of Concanavalin A; only Glucan #300 showed statistically the same effects.

Next, we focused on the use of glucan as an adjuvant. As an experimental model, we used immunization with ovalbumin. Glucans were applied together with two intraperitoneal doses of antigen, a commonly used Freund's adjuvant was used as additional positive control. The results (Table **6**) showed that despite the fact that all glucans potentiated the antibody response, significant differences between individual samples existed. The highest activity was measured in case of glucagel, Wellmune, Glucan #300 and Beta Right.

DISCUSSION

Despite the extensive amount of scientific reports about glucans and their biological activities, most of the studies are

Table 2. Effect of Dose on Stimulation of Phagocytosis

Dose (μg)	25	50	100	200	400	800
Maitake Gold	34.7 ± 2.4	35.5 ± 3.1	38.9 ± 3.1*	45.4 ± 4.1*	46.0 ± 4.0*	46.2 ± 4.2*
Krestin	36.2 ± 1.1	37.8 ± 2.1*	44.9 ± 2.5*	44.1 ± 3.5*	48.5 ± 4.4*	49.2 ± 3.9*
Aktival	31.1 ± 1.6	34.6 ± 2.0	38.9 ± 3.0*	40.2 ± 3.3*	41.1 ± 4.7*	40.5 ± 3.7*
Immutol	32.3 ± 2.1	35.5 ± 2.9	39.6 ± 3.3*	41.3 ± 4.1*	41.7 ± 3.5*	43.0 ± 4.1*
Now Glucan	36.2 ± 2.1	39.8 ± 1.1*	46.2 ± 2.5*	41.4 ± 3.0*	44.1 ± 3.6*	42.9 ± 3.8*
Sweet Beta Glucan	30.1 ± 2.2	31.2 ± 1.8	33.7 ± 2.3	34.1 ± 2.0	33.8 ± 2.4	37.8 ± 3.2*
Beta Right 101	35.1 ± 0.8	42.7 ± 2.7*	44.9 ± 3.0*	47.1 ± 3.3*	48.2 ± 3.8*	48.1 ± 2.1*
Epicor	30.1 ± 1.1	28.5 ± 3.2	30.6 ± 2.4	33.2 ± 3.3	35.6 ± 3.8	34.9 ± 4.6
NSC	29.9 ± 3.3	32.9 ± 2.5	31.6 ± 3.2	33.7 ± 3.8	35.7 ± 3.5	36.6 ± 2.9*
Glucagel	30.1 ± 1.7	32.5 ± 2.8	35.6 ± 2.1	37.1 ± 2.6*	36.9 ± 3.0*	38.0 ± 2.9*
Better Immunity	32.1± 0.8	33.6 ± 1.7	33.8 ± 3.0	35.5 ± 2.9	36.1 ± 4.2	34.2 ± 3.2
Macroforce	29.7 ± 3.0	30.6 ± 0.9	32.1 ± 2.8	33.6 ± 2.5	32.9 ± 5.1	33.8 ± 2.7
Solgar	30.1 ± 3.1	31.2 ± 2.2	32.5 ± 4.4	31.1 ± 2.4	34.8 ± 2.5	33.2 ± 2.9
Wellmune	28.5 ± 2.8	35.0 ± 2.1	42.1 ± 3.0*	44.2 ± 3.1*	44.7 ± 2.5*	45.9 ± 3.7*
Swanson	27.9 ± 1.2	30.1 ± 2.3	33.5 ± 2.7	35.2 ± 2.2	34.7 ± 1.9	35.9 ± 4.1
Glucan #300	44.1 ± 2.5*	48.8 ± 2.1*	55.7 ± 3.2*	56.1 ± 2.9*	55.9 ± 3.2*	60.9 ± 4.0*

Control values (PBS) were 30.4 ± 2.7. *Significant at <0.05 level.

Table 3. Effects of Glucan on Superoxide Anion Production

Glucan	superoxide anion	(nanomoles per 2.5 x 10^5 cells)	
	Mouse neutrophils	HL-60	U937
Maitake Gold	1.38 ± 0.22**	1.45 ± 0.33**	1.54 ± 0.25**
Krestin	1.17 ± 0.33**	1.02 ± 0.26**	1.11 ± 0.15*
Aktival	0.36 ± 0.09	0.27 ± 0.11	0.65 ± 0.23*
Immutol	0.67 ± 0.12*	0.67 ± 0.24*	1.02 ± 0.34*
Now Glucan	1.27 ± 0.09**	0.98 ± 0.11**	1.07 ± 0.22*
Sweet Beta Glucan	0.44 ± 0.07*	0.30 ± 0.06*	0.66 ± 0.15*
Beta Right	1.12 ± 0.15**	1.23 ± 0.16**	1.30 ± 0.25**
Epicor	0.49 ± 0.16*	0.34 ± 0.24*	0.45 ± 0.11
NSC	0.44 ± 0.15	0.35 ± 0.08*	0.71± 0.16*
Glucagel	1.49 ± 0.33**	1.50 ± 0.41**	1.43 ± 0.38**
Better Immunity	0.78 ± 0.12*	0.98 ± 0.16*	1.11± 0.18*
Macroforce	0.56 ± 0.20*	0.55 ± 0.09*	0.97± 0.24**
Solgar	0.37 ± 0.06	0.22 ± 0.04*	0.69 ± 0.17*
Wellmune	1.01± 0.23*	0.99 ± 0.18**	1.24 ± 0.20**
Swanson	0.87 ± 0.17*	0.25 ± 0.03*	0.77± 0.12*
Glucan #300	1.69 ± 0.34**	1.55 ± 0.27**	1.81 ± 0.35**
PBS	0.23 ± 0.07	0.11 ± 0.02	0.33 ± 0.06

*Significant stimulation of superoxide anion production at P<0.05 level.
**Significant stimulation of superoxide anion production at P<0.01 level.

Table 4. Effects of Glucan on Nitrite Oxide Production

Glucan	Nitrite oxide	(μmol/L)	
	Mouse neutrophils	HL-60	U937
Maitake Gold	4.11 ± 0.11	3.98 ± 0.23	5.79 ± 0.43
Krestin	5.27 ± 0.78	4.98 ± 0.66	5.56 ± 1.11
Aktival	5.04 ± 0.57	3.87 ± 0.87	5.52 ± 0.54
Immutol	2.99 ± 0.77	3.68 ± 0.54	4.61± 0.99
Now Glucan	5.99 ± 0.68	6.01± 1.12	7.21 ± 1.07
Sweet Beta Glucan	2.88 ± 0.44	4.01 ± 0.95	4.24 ± 1.01
Beta Right	4.61 ± 0.87	5.55 ± 0.99	6.12 ± 1.22
Epicor	4.33 ± 1.54	3.75 ± 0.89	4.87 ± 0.98
NSC	3.78 ± 0.66	3.86 ± 0.75	4.44 ± 1.04
Glucagel	6.46 ± 1.06	5.15 ± 0.78	6.69 ± 1.25
Better Immunity	2.98 ± 1.43	3.78 ± 1.01	4.99 ± 0.98
Macroforce	3.56 + 0.55	4.72 ± 0.87	5.01 ± 0.99
Solgar	4.55 ± 1.07	4.88 ± 1.01	5.11 ± 1.27
Wellmune	7.01± 1.77	5.36 ± 0.79	6.98 ± 1.21
Swanson	4.22 ± 0.99	4.87 ± 2.02	4.98 ± 1.78
Glucan #300	7.87 ± 1.55	6.26 ± 1.77	8.37 ± 2.03
PBS	0.24 ± 0.04	0.09 ± 0.02	0.31 ± 0.05

Stimulation of superoxide anion production was significant at P<0.01 level.

Table 5. Effect of Glucan on Secretion of IL-2

Glucan	IL-2 (pg/ml)
Maitake Gold	695.7± 23.9
Krestin	601.2 ± 109.5
Aktival	445.7 ± 95.7
Immutol	326.5 ± 99.6
Now Glucan	525.6 ± 109.5
Sweet Beta Glucan	237.3 ± 67.4
Beta Right	678.1± 156.3
Epicor	113.8 ± 27.8
NSC	267.1 ± 50.5
Glucagel	206.7 ± 56.4
Better Immunity	156.0 ± 33.9
Macroforce	358.9 ± 87.1
Solgar	89.9 ± 33.4
Wellmune	711.2 ± 167.5
Swanson	177.8 ± 36.6
Glucan #300	983.9 ± 122.8
Con A	1 047.6 ± 287.7

All glucans showed significant stimulation of IL-2 secretion at P<0.01 level.

Table 6. Effects of Glucans on Formation of Antibodies Against Ovalbumin

Glucan	% of control
Maitake Gold	211.7± 20.1*
Krestin	181.3 ± 39.1
Aktival	85.3 ± 17.3
Immutol	136.2 ± 33.0
Now Glucan	215.4 ± 50.1*
Sweet Beta Glucan	33.6 ± 7.2
Beta Right	278.9± 43.7*
Epicor	38.1 ± 7.0
NSC	137.7 ± 20.8
Glucagel	396.5 ± 50.2*
Better Immunity	247.1 ± 29.5*
Macroforce	143.8 ± 25.3
Solgar	55.6 ± 13.0
Wellmune	322.6 ± 47.2*
Swanson	111.4 ± 14.4
Glucan #300	343.9 ± 43.1*
Ovalbumin	164.2 ± 28.5
Ovalbumin + FA	493.8 ± 57.4*

FA – Freud's adjuvant.
*Significant at <0.05 level.

focused either on the description of newly isolated glucans or on the description of their biological activities. Comprehensive reviews comparing several glucans are rare. In one of those, Yadomae reviewed how structural properties of glucans affected biological activities and found that branched or linear 1,4 glucans have limited activity and βglucans with a 1,3 configuration with additional branching at the position 0-6 of the 1-3 linked D-glucose residues have the highers immunostimulating activity [15]. Readers seeking additional reviews might look up Kogan [16] or Vetvicka [17]. However, it is important to keep in mind that these reviews are oriented towards comparing results of numerous publications and none of them offers a one-on-one comparison of several glucans. Very few studies tried to directly compare individual glucans and the number of used glucans was seriously limited [5, 7-8].

Glucans are well known to stimulate cellular immunity, thus phagocytosis is one of the first tests of the immunological characteristics of any glucan. We used the 2-hydroxyethyl methacrylate particles that have only a slight negative charge and as a result do not nonspecifically adhere to the cell surface [18]. This guarantees that only phagocytosing cells will engulf these particles and significantly lowers the chance of false negativity. Our investigation showed that while most of the tested glucans to some extent stimulated phagocytosis of synthetic microspheres, these effects were usually nonsignificant or achieved only using the highest dose.

Ligand-receptor interactions during phagocytosis result in a substantial outburst of metabolic activity. During these initial stages of endocytosis, the phagocytes exhibited a large increase in oxygen consumption, hexosemonophosphate shunt activity, and production of active oxygen species. This process is known as the oxidative burst. Production of active oxygen molecules is necessary for the destruction of invading microorganism. As several glucans have been shown in past to stimulate oxidative burst [19-21], we evaluated our glucan on stimulation of superoxide anion and nitrite oxide.

In addition to the direct effect on various cells of the immune system, the immunostimulating action of β-glucans is caused by potentiation of a synthesis and release of several cytokines such as TNFα, IFNγ, IL-1 and IL-2. This cytokine stimulating activity is thought to be dependent on the triple helix conformation [22]. The only glucan without a trace of pro-inflammatory cytokine stimulation is PGG-glucan [23]. We focused on the stimulation of IL-2 production by spleen cells *in vitro* and found that whereas all glucans significantly stimulated production of IL-2, only one of the samples (Glucan #300) showed stimulation comparable to the common stimulator Concanavalin A. The activity of the most active glucan was comparable to the previously published data [6, 13].

Glucans are usually considered stimulators of the cellular branch of immune reaction and only limited attention has been focused on their potential effects on antibody response. However, recent studies showed that glucan not only potentiated antibody response [24], but can be used for robust stimulation of both cellular and humoral immune responses during vaccination [25]. Rather surprisingly, our results showed that all of the tested glucans revealed some level of stimulation of antibody response, the strongest being Glu-

cagel T and Glucan #300. However, the stimulation was always significantly lower than in the case of combining antigen and Freund's adjuvant.

Data presented in this study clearly demonstrated significant differences in activities among individual types of glucans. In addition, it is clear that individual glucans can be highly active in one particular part of immune reaction (e.g., Glucagel T on antibody production), and mediocre in other parts of immune reaction. Glucan #300 showed the highest activity in all tested reactions (with the exception of the antibody formation where it was close second). Some of the tested glucans (Beta Right 101, Swanson and Glucan #300) case from the same manufacturer, but still strongly differed in their immunostimulating activities. The most probable explanation is the purity of these glucans – Glucan #300 has purity over 85%, whereas Beta Right 101 and Swanson have purity of app. 70 percent.

CONCLUSION

Several conclusions can be made: 1) not all glucans are created equal; 2) some of the commercial glucans have surprisingly low activity; 3) most glucans strongly differ in biological effects based on tested characteristics, and 4) no clear relevance between the source of glucan and its activity has been found. Also, another important point is the fact that some of the glucans exhibited low activity. In these cases, however, you need up to 100x more of glucan to illicit high activity. This means that the low activity is not caused by a lower percentage of glucan but, rather, that the glucans with limited biological activities will not be comparable to the "better" glucans regardless of the dosage.

CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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