

DRAFT

# An Evaluation of the Immunological Activities of Commercially Available Beta 1, 3 Glucans

Vaclav Vetvicka, PhD\*, Jana Vetvickova, MS  
University of Louisville, Department of Pathology, Louisville, KY

## ABSTRACT

### Introduction

$\beta$ 1,3-Glucan's role as a biologically active immunomodulator has been well documented for over 40 years. Interest in the immunomodulatory properties of polysaccharides was initially raised after experiments showing that a crude yeast cell preparation stimulated macrophages via activation of the complement system.<sup>1</sup> Further work identified the immunomodulatory active component as  $\beta$ 1,3-glucan.<sup>2</sup> Numerous studies (currently more than 1,600 publications) have subsequently shown that  $\beta$ 1,3-glucans, either particulate or soluble, exhibit immunostimulating properties, including antibacterial and anti-tumor activities.<sup>3,4</sup>

Despite extensive investigations, no consensus on the source, size and other biochemical or physicochemical properties of  $\beta$ 1,3-glucan has been achieved. In addition, numerous concentrations and routes of administration have been tested - including oral, intraperitoneal, subcutaneous and intravenous applications.

---

\* Correspondence:  
Vaclav Vetvicka, PhD.

This fact, together with the fact that there are probably more than a hundred different samples on the U.S. market alone, leads to confusion about the quality, biological effects and overall efficiency of glucan. Therefore, we decided to compare the basic immunological activities of a group of glucans. The list of products chosen came from those heavily advertised, commonly available and easily obtained in the US, Europe, Southeast Asia and Japan. In order to be certain that we are measuring the effects of glucan only, we picked the commercial samples with glucan (either from one source or a mixture of different glucans) as the only active ingredient.

The collection of tested biological reactions (phagocytosis, surface markers on splenocytes, cytokine synthesis and stimulation of antibody response) represents both the humoral and cellular branches of the immune reaction, thus offering insight as to the immunological activities of studied glucans.

## MATERIAL AND METHODS

### Animals

Female, 6 to 10 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<sub>2</sub> asphyxiation.

### Materials

RPMI 1640 medium, sodium citrate, dextran, Ficoll-Hypaque, antibiotics, sodium azide, bovine serum albumin (BSA), Wright stain, Limulus lysate test E-TOXATE,

Freund's adjuvant and Concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT).

### $\beta$ -1,3 glucans

The glucans used in this study were purchased from the following companies: Now BETA glucan from Now Foods (Bloomington, IL), IMMUTOL from Biotec (Tromsø, Norway), Immune Builder and Maitake Gold 404 from Mushroom Science (Eugene, OR), Glucan #300 from Transfer Point (Columbia, SC), Glucagel T from GraceLinc (Christchurch, New Zealand), and Senseiro from Sundory (Tokyo, Japan).

### Antibodies

For fluorescence staining, the following antibodies have been employed: anti-mouse CD4, CD8 and CD19, conjugated with FITC were purchased from Biosource (Camarillo, CA).

### Flow cytometry

Cells were stained with monoclonal antibodies on ice in 12 x 75-mm glass tubes using standard techniques. Pellets of  $5 \times 10^5$  cells were incubated with 10  $\mu$ l of FITC-labeled antibodies (1 to 20  $\mu$ g/ml in PBS) for 30 minutes on ice. After washing with cold PBS, the cells were re-suspended in PBS containing 1% BSA and 10 mM sodium azide. Flow cytometry was performed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer and the data from over 10,000 cells/samples were analyzed.

### Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier.<sup>5,6</sup> Briefly: peritoneal cells were incubated with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA;  $5 \times 10^8$ /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. The same smears were also used for evaluation of cell types.

### Evaluation of IL-2 production

Purified spleen cells ( $2 \times 10^6$ /ml in RPMI 1640 medium with 5% FCS) were added into wells of a 24-well tissue culture plate. After addition of 1 mg of Concanavalin A into positive-control wells, cells were incubated for 72 hrs. in a humidified incubator (37°C, 5% CO<sub>2</sub>). At the endpoint of incubation, supernatants were collected, filtered through 0.45 mm filters and tested for the presence of IL-2.<sup>7</sup> Levels of the IL-2 were measured using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

## 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, we tried to include both soluble and insoluble glucans, and also glucans from different sources, including yeast, mushrooms and cereals.

Glucagel barley  $\beta$ -glucan is a mixed link (1,3, 1,4)- $\beta$ -D-glucose polymer, in which cellotriosyl and cellotetraosyl residues occur in a ratio of ~3:1. The natural purification process yields a reduced molecular weight  $\beta$ -glucan (typically ~130 kDa) that is more readily hydrated than other conventionally purified  $\beta$ -glucans. The typical carbohydrate content is 85-90%.

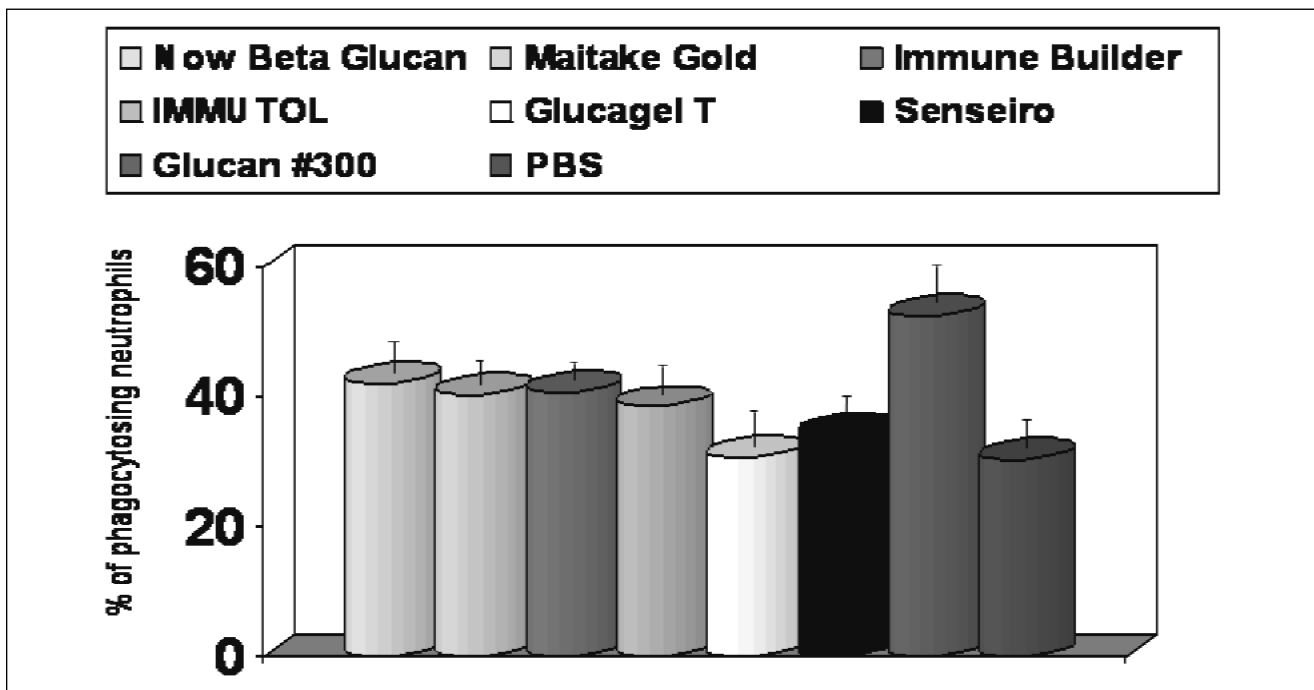
Senseiro is a soluble, high molecular weight glucan isolated from *Agaricus blazei*, consisting of approximately 63% carbohydrate. Glucan #300 is a proprietary (1,3, 1,6)- $\beta$ -D-glucan purified from *Saccharomyces cerevisiae* by Biothera for Transfer Point and even when corresponding to the glucan sold under WGP name, has much higher purity (app. over 96%).

$\beta$ -Glucans are generally considered to be potent stimulators of cellular immunity, with macrophages and neutrophils being the most important targets. Not surprisingly, we started our evaluation of glucan activities by phagocytosis. We used the synthetic polymeric microspheres, HEMA, since their use, dose and timing are already well established in glucan studies.<sup>7-9</sup> Results summarized in Figure 1 show significant effects of glucan samples on encapsulation of synthetic particles by peripheral blood neutrophils. The significant stimulation of phagocytic activity was found with five glucans – Now Beta Glucan, Maitake Gold, Immune Builder, IMMUTOL and Glucan #300. The other samples, with the exception of Glucagel T, also stimulated the phagocytosis, but at a much lower level and the results were not significant.

Next, we compared the effects of tested glucans on the expression of several membrane markers on splenocytes. Twenty-four hours after an ip. injection of 100  $\mu$ g of individual glucan, spleen cells were isolated and the surface expression of CD4, CD8 and CD19 was evaluated by flow cytometry. The results summarized in Figure 2 demonstrated that only three glucans - Now Beta Glucan, Maitake Gold, and Glucan #300 significantly increased the migration of CD4- and CD8-positive T lymphocytes; none of the glucan had any significant effect on changes in presence of CD19-positive B lymphocytes.

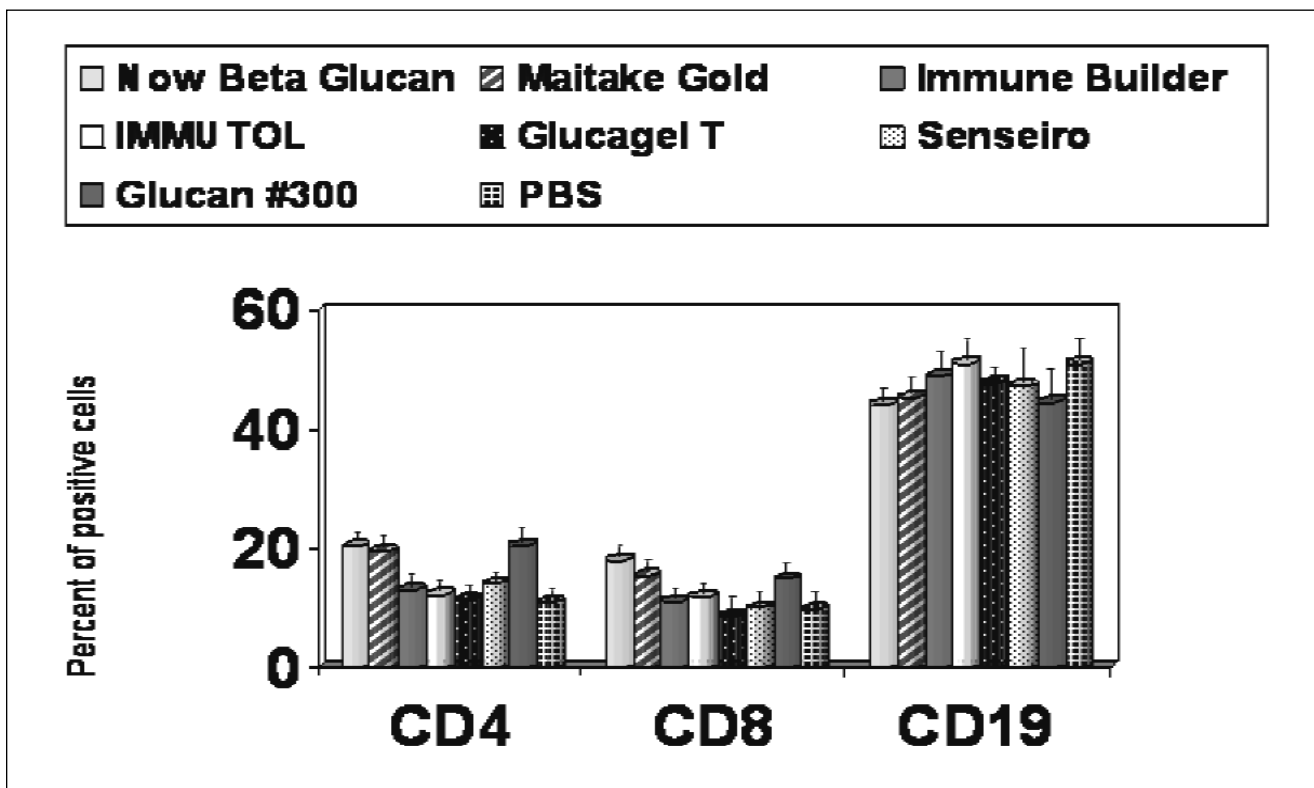
Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by spleen cells (Figure 3). The production of IL-2 was measured after a 72 hr. *in vitro* incubation of spleen cells isolated from control and glucan-treated mice. Again, treatment of mice with Now Beta Glucan, Maitake Gold, and Glucan #300 showed the highest stimulation of IL-2 production. Immune Builder and IMMUTOL showed medium

Figure 1.



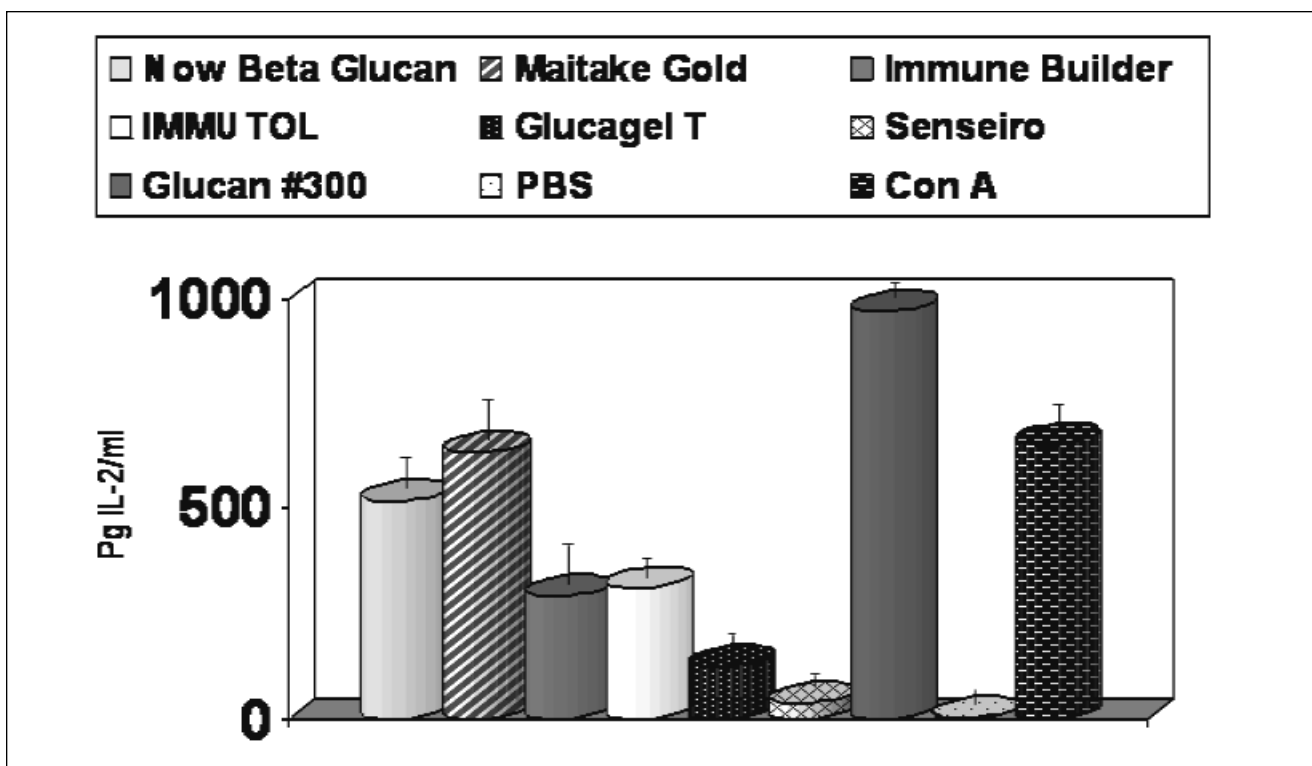
Effect of an ip. administration of 100 µg of different glucan samples on phagocytosis by peripheral blood granulocytes. Each value represents the mean ± SD. \*Represents significant differences between control (PBS) and glucan samples at P ≤ 0.05 level.

Figure 2.



Effect of ip. injection of 100 µg of tested glucans on the expression of CD4, CD8 and CD19 markers by spleen cells. The cells from three donors at each time interval were examined and the results given represent the means ± SD. \*Represents significant differences between control (PBS) and samples at P ≤ 0.05 level.

Figure 3.



Effects of glucans on Con A-stimulated secretion of IL-2  $\beta$  by spleen cells. \*Represents significant differences between control (PBS) and samples at  $P \leq 0.05$  level.

level stimulation. As the secretion of IL-2 by non-stimulated splenocytes (PBS group) is almost zero, even low stimulation by Glucagel T was significant. Another way to compare the effect on IL-2 formation and/or secretion is to compare it to the Con A stimulation.. In this case, only Glucan #300 showed higher effects than Con A, whereas Now Beta Glucan and Maitake Gold were comparable, and the rest of the glucans show much smaller effects.

We then 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 (Figure 4) showed that only Immune Builder and Senseiro glucans were without any effects on antibody response. All other glucans significantly supported the formation of specific antibodies. Glucans with the highest stimulation were Glucagel T and Glucan #300. It must be noted, however, that none of the glucans potentiated the humoral immunity to the level of Freund's adjuvant.

Table 2 summarizes the activities of individual glucans in all tested functions. Clearly, the most active samples were Glucan #300, followed by Now Beta Glucan and Maitake Gold 404. Senseiro glucan was almost without any measurable activity.

## DISCUSSION

Despite the extensive amount of scientific reports about glucans and their biological activities, most of the studies are focused on the description of chemical and/or biological properties of one particular glucan. Numerous types of glucans have been isolated from almost every species of yeast and fungi. For a long time, attention was focused mainly on glucans isolated from yeast and mushrooms. Recently, the existence of a highly purified linear  $\beta$ -1,3 glucan named Phycarine, and subsequent study showing that Phycarine induced a broad range of defense reactions in tobacco cells,<sup>10</sup> brought new attention to seaweed-derived glucans.<sup>11-13</sup> More studies revealed that Phycarine significantly stimulated phagocytosis, synthesis and release of IL-1, IL-6 and TNF- $\alpha$ , and NK cell-mediated killing of tumor cells both *in vitro* and *in vivo*.<sup>8</sup> Similarly, recent clinical trials demonstrated the high activity of glucan isolated from barley.<sup>14</sup> It is clear, therefore, that the biological activities of glucans might be related more to the purity and biochemical/physiochemical characteristics than to the source.

Comprehensive reviews comparing several glucans are rare. However, in one of those studies, Yadomae reviewed how the structural properties of glucans affected biological activities and found that branched or linear 1,4 glucans have

**Table 1.**

<b>Glucan used in this study</b>			
<b>Name</b>	<b>Source</b>	<b>Manufacturer/Distributors</b>	<b>Solubility</b>
β-1,3/1,6-D-glucan	<i>Saccharomyces cerevisiae</i> <i>Grifola frondosa</i>	Now Foods	No
MaitakeGold 404	<i>Grifola frondosa</i>	MushroomScience	Yes
Immune Builder	<i>Agaricus blazei</i> <i>Cordyceps sinensis</i> <i>Coriolus versicolor</i> <i>Ganoderma lucidum</i> <i>Lentinula edodes</i> <i>Grifola frondosa</i>	MushroomScience	No
IMMUTOL	<i>Saccharomyces cerevisiae</i>	Biotec ASA	No
Glucagel T	Barley	GraceLinc	
Senseiro	<i>Agaricus blazei</i>	Sundory	Yes
Glucan #300	<i>Saccharomyces cerevisiae</i>	Transfer Point	No

**Table 2.**

<b>Comparison of individual glucans</b>				
<b>Name</b>	<b>Phagocytosis</b>	<b>CD expression</b>	<b>IL-2 production</b>	<b>Antibody formation</b>
Now Beta Glucan	++	+++	++	++
MaitakeGold 404	++	+++	++	++
Immune Builder	++	+	+	-
IMMUTOL	++	+	+	+
Glucagel T	-	-	+/-	+++
Senseiro	+	+	-	-
Glucan #300	+++	+++	+++	+++

limited activity and β-glucans with a 1,3 configuration with additional branching at the position 0-6 of the 1-3 linked D-glucose residues have the highest immunostimulating activity.<sup>15</sup> Readers seeking additional reviews might see Kogan<sup>16</sup> or Vetvicka.<sup>17</sup> 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 face-to-face comparison of several glucans. At the same time, with the high number of individual glucans and huge differences in their biological activities, it is imperative to evaluate their

biological properties before any suggestions for use of a particular glucan can be made.

In our paper, we compared seven commercially successful glucans, differing both in source (mushroom, yeast and barley) and solubility. At the same time, we used identical amounts of glucans from each sample. In the case of complex mixtures (such as Immune Builder), the total amount of used sample corresponded to the ratio of individual glucans.

As various glucans are well known to stimulate phagocytosis,<sup>18</sup> one of the first tests of the immunological characteristics of any glucan is phagocytosis. We used the 2-hydroxyethyl methacrylate particles, which have only a slight negative charge and thus do not nonspecifically adhere to the cell surface. This guarantees that only phagocytosing cells will engulf these particles and significantly lowers the chance of false negativity.<sup>19</sup> Our investigation showed that while most of the tested glucans stimulated phagocytosis of synthetic microspheres (with the exception of Glucagel T), the highest effects were obtained with Glucan #300.

As some of the glucans are known to regulate the influx of cells into the individual lymphatic organs,<sup>8</sup> we compared the effects of a single injection on expression of the basic membrane markers present on splenocytes. Only three glucans - Now Beta Glucan, Maitake Gold, and Glucan #300 - changed the number of CD4- and CD8-positive lymphocytes. No glucan significantly changed the percentage of B lymphocytes. The effects on CD4-positive cells corresponded to the previously found effects of Phycarine<sup>8</sup> or lentinan.<sup>20</sup>

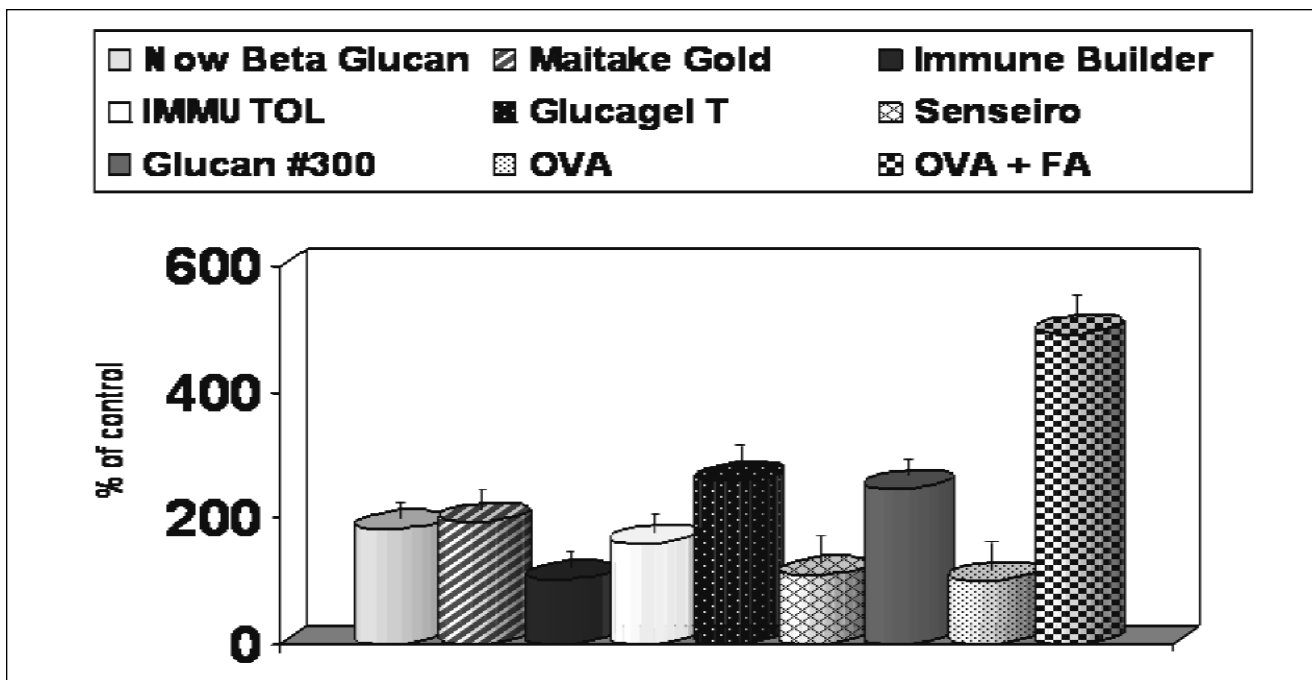
In addition to the direct effect on various cells of the immune system, the immunostimulating action of  $\beta$ -glucans is caused by potentiation of a synthesis and release of several cytokines such as TNF $\alpha$ , IFN $\gamma$ , IL-1 and IL-2. This cytokine stimulating activity is dependent on the triple helix conformation.<sup>21</sup> The only glucan without a trace of pro-

inflammatory cytokine stimulation is PGG-glucan.<sup>22</sup> We focused on the stimulation of IL-2 production by spleen cells *in vitro* and found that whereas all glucans (with the exception of Senseiro) stimulated production of IL-2, only two of the samples (Maitake Gold and 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.<sup>9,23</sup>

Glucans are usually considered stimulators or modulators of the cellular branch of immune reaction and very little attention has been focused on their potential effects on antibody response. We decided to take advantage of the recently published method of evaluating the use of glucan as adjuvant.<sup>24</sup> Our results rather surprisingly showed that most of the tested glucans revealed some level of stimulation of antibody response, the strongest being Glucagel T and Glucan #300. In this case, however, the stimulation was always significantly lower than in the case of combining antigen and Freund's adjuvant.

Data presented in this study and summarized in Table 2 clearly demonstrated the differences in activities among individual types of glucans. Also, it is clear that individual glucans can be highly active in one particular part of immune reactions (e.g., Glucagel T on antibody production), and almost without any significant biological activity in other parts of defense reaction. Glucan #300 showed not only a broad range of action, but in all tested reactions (with

Figure 4.



Effects of two ip. injections of tested glucans on formation of antibodies against ovalbumin. Mice were injected twice (two weeks apart) and the serum was collected 7 days after last injection. Level of specific antibodies against ovalbumin was detected by ELISA. As positive control, Freund's adjuvant was used. \*Represents significant differences between control (ovalbumin alone) and samples at  $P \leq 0.05$  level.

the exception of the antibody formation where it was the second most active sample) was the biologically most relevant immunomodulator.

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 differ in biological effects based on tested characteristics; and 4) for good results in immunomodulation, it is more imperative to find a glucan from a solid vendor who is able to back the claims with solid scientific data. Thinking about the biological source of glucan is much less important.

## REFERENCES

1. Benacerraf B, Sebastyen MM. Effect of bacterial endotoxins on the reticuloendothelial system. *Fed Proc.* 1957;16: 860-867.
2. Rigi SJ, Di Luzio NR, Identification of a reticuloendothelial stimulating agent in zymosan. *Am J Physiol.* 1961;200:297-300.
3. Di Luzio NR, Williams DL, McNamee RB, Edwards BF, Kitahama A. Comparative tumor-inhibitory and anti-bacterial activity of soluble and particulate glucan. *Int J Cancer* 1979; 24:773-779.
4. Mimura H, Ohno N, Suzuki I, Yadomae T. Purification, antitumor activity, and structural characterization of  $\beta$ -1,3-glucan from *Peiza vesiculosa*. *Chem Pharm Bull.* 1985; 33:5096-5099.
5. Vetvicka V, Fornusek L, Kopecek J, Kaminkova J, Kasperek L, Vranova M. Phagocytosis of human blood leukocytes: A simple micromethod. *Immunol. Lett.* 1982;5:97-100.
6. Vetvicka V, Holub M, Kovaru H, Siman P, Kovaru F. Alpha-feto-protein and phagocytosis in athymic nude mice. *Immunol Lett.* 1988;19:95-98.
7. Vetvicka V, Terayama K, Mandeville R, Brousseau P, Kournikakis B, Ostroff G. Pilot study: orally administered yeast  $\beta$ 1,3-glucan prophylactically protects against anthrax infection and cancer in mice. *JANA.* 2002;5:1-5.
8. Vetvicka V, and Yvin J.-C. Effects of marine  $\beta$ -glucan on immune reaction. *Int Immunopharmacol.* 2004; 4:721-730.
9. Vetvicka V, Vetvickova J. Immunostimulating properties of two different  $\beta$ -glucans isolated from Maitake mushrooms (*Grifola frondosa*). *JANA*,2005; 8:33-39.
10. Klarzynski O, Plesse BB, Joubert JM, Yvin J-C, Kopp M, Kloreg B, Fritig B. Linear beta-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiol.* 2000; 124: 1027-1038.
11. Pang ZC, Otake K, Maoka T, Hidaka K, Ishijima S, Oda M, Ohnishi M. Structure of beta-glucan oligomer from laminarin and its effect on human monocytes to inhibit the proliferation of U937 cells. *Biosci Biotech Biochem.* 2005; 69:553-558.
12. Kurashige S, Akuzawa Y, Endo F. Effects of *Lentinus edodes*, *Grifola frondosa* and *Pleurotus ostreatus* administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, N-butyl-N- butanolnitrosoamine. *Immunopharmacol Immunotoxicol.* 1997; 19:175-183.
13. Jamois F, Ferrieres V, Guegan JP, Yvin J-C, Plusquellec D, Vetvicka V. Glucan-like synthetic oligosaccharides - Iterative synthesis of linear oligo- $\beta$ -(1,3)-glucans and immunostimulatory effects. *Glycobiology* 2004; 15:393-407.
14. Cheung NK, Modak S. Oral (1 $\rightarrow$ 3),(1 $\rightarrow$ 4)-beta-D-glucan synergizes with antiganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma *Clin Cancer Res.* 2002; 8:1217-1223.
15. Yadomae, T. Structure and biological activities of fungal  $\beta$ -1,3-glucans. *Yakugaku Zasshi.* 2000; 120: 413-431.
16. Kogan G. in: A. Atta-ur-Rahman (Ed.), *Studies in Natural Products Chemistry.* Elsevier, Amsterdam, 2000.
17. Vetvicka V.  $\beta$ -Glucans as immunomodulators. *JANA.* 2001; 3:31-34.
18. Abel G, Szolosi J, Chihara G, Fachel J, Effect of lentinan and mannan on phagocytosis of fluorescent latex microbeads by mouse peritoneal macrophages: a flow cytometric study. *Int J Immunopharmacol.* 1989; 11:615-621.
19. Vetvicka V, Fornusek L, Polymer microbeads in immunology. *Biomaterials* 1987; 8:341-345.
20. Arinaga S, Karimine N, Takamuku K, Nanbara S, Nagamatsu M, Ueo H, Akiyoshi T. Enhanced production of interleukin 1 and tumor necrosis factor by peripheral monocytes after lentinan administration in patients with gastric carcinoma. *Int J Immunopharm.* 1992; 14:43-47.
21. Falch BH, Espevik T, Ryan L, Stokke BT, The cytokine stimulating activity of (1-3)- $\beta$ -D-glucans is dependent on the triple helix conformation. *Carbohydrate Res.* 2000; 329:587-596.
22. Bleicher P, Mackin W. Betafectin PGG-Glucan: A novel carbohydrate immunomodulator with anti-infective properties. *J Biotechnol Healthcare* 1995; 2:207-222.
23. Vetvicka V, Terayama K, Mandeville R, Brousseau P, Kournikakis B, Ostroff G. Pilot study: orally administered yeast  $\beta$ 1,3-glucan prophylactically protects against anthrax infection and cancer in mice. *JANA* 2002; 5: 1-5.
24. Vetvicka V, Dvorak B, Vetvickova J, Richter J, Krizan J, Sima P, Yvin J-C. Orally-administered marine  $\beta$ 1,3 glucan Phycarine stimulates both humoral and cellular immunity. *Int J Biol Macromol.* in press.