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Comparison of immunological effects of commercially available β-glucans

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Abstract

Biological and most of all immunological effects of natural immunomodulator glucan are already well established. However, since hundreds of individual glucans, isolated from various sources, used at different concentrations and having different physicochemical characteristics are being used, the current scientific knowledge is not complete. In addition, direct comparisons of individual glucans are quite rare. In the present paper, we tested fifteen varieties of glucans differing in source and solubility. Whereas no direct connection between source and immunological effects was found, we can conclude that the best glucans have pleiotropic effects stimulating all facets of immunological reactions, whereas other glucans have low effects or none at all.

Keywords: Glucan, phagocytosis, IL-2, antibodies, breast cancer, superoxide anion

Introduction

β1, 3-D-glucans (hereafter referred to as glucans) form part of a group of natural biologically active compounds generally called biological response modifiers. These molecules are highly conserved carbohydrates forming structural components of cell walls of yeast, fungi, seaweed, and cereals. Generally, the term glucan is some times used as a chemical name of glucose polymer and represents a group of chemically heterogeneous carbohydrates consisting of various numbers of glucose molecules bound together in several types of linkages.

The history of glucan began over 50 years ago with two different starting points—one originated in Europe and the United States and the second in Japan. Research on glucans in the Euro-American milieu was based on the immunomodulatory effects of zymosan (mixture of polysaccharides isolated from the cell walls of *Saccharomyces cerevisiae*). On the other hand, the Japanese research was based on Asian medicine, where consuming medicinal mushrooms (such as shiitake or reishi) has been a long tradition.

The biological effects of glucans are already well established and reach from stimulation of anti-infectious immunity to potentiation of cancer defense, from stress reduction to reduction of cholesterol (for review see [1,2]). In addition to various animal studies, where glucans were found to be active in wide range of species, basically from shrimp to horses, the effects of glucans have also been also examined in human models. Soluble glucan was found to decrease the infection incidence and need for antibiotics [3]. Recently, glucan was successfully used as part of a vaccine for high risk neuroblastoma [4]. In addition, a series of clinical studies showed strong effects on the treatment of children with chronic respiratory problems [5,6]. In Japan, glucan has been widely used, since 1983, in the treatment of gastrointestinal cancer [7].

Over 7,000 publications describing various biological effects of glucans can be found in scientific literature. One of the problems resulting in low acceptance of glucans in current medicine 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 physicochemical characteristics. Diverse data on the comparison of structure, molecular size, and biological effects can be found in the literature [2]. Some studies suggest that the effects are dependent on the helical conformation [8]. However, the triple helix structure most likely is not a solely effective form of glucan, because alkaline treatment, used in most isolation procedures, destroys this structure [9].

In addition, various concentrations and routes of administration (oral, intraperitoneal, intravenous, subcutaneous) have been tested. All this leads to severe 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 [10-15], with only one really comprehensive study being published during last 5 years [16]. This led us to the current comparative review of 15 different commercially available glucans.

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 CO_2 asphyxiation followed by cervical dislocation.

Material

All glucans were either donated or purchased from the manufacturers or distributors as shown in **Table 1**.

Cell lines

Human myeloblastic cell line HL-60 was obtained from the ATCC (Manassas, VA). The BALB/c mouse-derived mammary tumor cell line Ptas 64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, Detroit, MI. The cells 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% CO_2/95% air incubator.

Tumor inhibition *in vivo*

Mice were injected directly into their mammary fat pads with $1x10^6$/mouse of Ptas64 cells in PBS. The experimental treatment was begun after palpable tumors were found (app. 14 days after injection of cells) and after mice were assigned to experimental groups. Experimental treatment was achieved by intraperitoneal injections of tested samples diluted in PBS (once/day for 14 days). After treatment, the mice were sacrificed, tumors removed and weighed [17]. These experiments were repeated three times with 3 mice per each group.

Phagocytosis

Phagocytosis of synthetic polymeric microspheres was described earlier [18]. Briefly: 0.1 ml of peripheral blood from mice injected with various doses of glucan or PBS was incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml). The tubes were incubated at 37°C for 60 min., with intermittent shaking. Smears were stained with Wright stain (Sigma). The cells with three or more HEMA particles were considered positive. Mice were injected with either glucan or PBS (control). All experiments were performed in triplicate. At least 300 cells were examined in each experiment.

IL-2 secretion

Purified spleen cells ($2x10^6$/ml in RPMI 1640 medium with 5% FCS) obtained from mice injected with 100 mg glucan or PBS were added into wells of a 24-well tissue culture plate. Cells were incubated for 48 hrs in a humidified incubator (37°C, 5% CO_2/95% air). Addition of 1 mg of Concanavalin A (Sigma) was used as a positive control. At the endpoint of incubation, supernatants were collected, filtered through 0.45 mm filters and tested for the presence of IL-2 using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

Antibody formation

The technique was described earlier [16]. Briefly: formation of antibodies was evaluated using ovalbumin (Sigma) as an

Table 1. Types of glucan used.

Glucan	Source	Solubility	Manufacturer
Beat Max	Yeast	Insoluble	Chisolm Biological Laboratories, Aiken, SC
Oat Beta Glucan	Oat	Insoluble	Health Breakthroughs, Lake Oswego, OR
Bio-Glucan	Yeast	Insoluble	Pharma Nord, Vojens, Denmark
Qore defense	Mushroom	Insoluble	Quivana, Provo, UT
Immunox 3-6	Yeast	Insoluble	Xymogen, Orlando, FL
Betacan 500	Yeast	Insoluble	Arrowhead Healthworks, Cedarpine Park, CA
Glucan Real	Mushroom	Soluble	QueGen Biotech, South Korea
MC-Glucan	Mushroom	Soluble	Macrocare Tech, South Korea
Beta Glucan (Germany)	Yeast	Insoluble	Biotikon, Germany
Barley Glucan	Barley	Insoluble	Sigma, St. Louis, MO
Beta Glucan	Mushroom/yeast	Partly soluble	Vitabase, Monroe, GA
Reishi	Mushroom	Soluble	Hostdefence, Olympia, WA
Beta Glucan	Yeast	Insoluble	Greenpath, Wrightsville Beach, NC
Hliva ustricna	Mushroom	Insoluble	Walmark, Trinec, Czech Republic
Glucan #300	Yeast	Insoluble	Transfer Point, Columbia, SC

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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 Freunds adjuvant (Sigma) was used.

Superoxide production

Mouse neutrophils were isolated using Ficoll-Hypaque separation as described [19]. Cells (either peripheral blood neutrophils or HL-60 cell line) were incubated in a final volume of 200 µl of medium containing 0.1% gelatin and 100 µM cytochrome C (Sigma). Mice were challenged with 100 µg of individual glucans 24 hrs earlier. Cells were incubated with 1 µg/ml of glucans for 24 hrs. For the superoxide production, the reaction was initiated by the addition of 5 ng/ml PMA (Sigma). Incubation was terminated by rapid cooling the cells. Superoxide production was quantitated by measuring the reduction of cytochrome c (Type VI, Sigma, 100 nmol/tube). 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/2.5x10^5 cells/30 minutes, after subtraction of the superoxide dismutases and spontaneous release controls [19].

IFNγ production

Twenty four hours after ip. injection with 100 µg of glucan, 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 [14].

Results

Glucans are manufactured, tested and used in almost every country of the world. For our study, we decided to use several samples differing in the source (yeast, mushroom, oat and barley), solubility (both soluble and insoluble), and origin (United States, Germany, Denmark, South Korea and Czech Republic). All of these glucans are commercially available, often in several countries. Basic information about individual types of glucan and their manufacturers or distributors are given in **Table 1**. Almost none of the manufacturers provide any information about solubility. We tested the solubility by solubilization of three different concentrations of glucan in water at 22°C under constant shaking for 30 minutes. Based on the amount of sugar measurable in the solution after filtration (data not shown), we called the sample soluble (over 90% of glucan), semisoluble (20-89%) or insoluble (below 20%).

The effects of glucans on cellular immunity are well established. Usually, the test of choice are the effects on phagocytosis, as if the glucan does not stimulate phagocytosis, it might have little effects on additional facets of the defense reactions. As in our previous comparative study, we employed synthetic hydroxyethyl methacrylate particles [16] known for minimal nonspecific adhesion to the membrane of phagocytosing cells [20]. We injected the mice with different doses of glucan and 24 hrs later tested the effects of glucans on phagocytic ability of peripheral blood neutrophils. Data shown in **Table 2**

Table 2. Effects of various glucans on phagocytosis.

Dose (mg)	25	50	100	200	400	800
BetaMax	33.1±2.9	30.9±3.1	35.1±4.2	37.7±3.2*	38.8±3.5*	37.9±2.9*
Oat Beta Glucan	30.7±1.9	32.5±2.7	34.1±2.8	33.6±2.6	35.5±3.7	36.2±2.8
Bio-Glucan	32.5±2.6	34.1±2.5	38.8±3.1*	40.2±3.0*	42.8±3.3*	44.1±3.1*
Qore defense	30.9±2.2	33.7±2.9	34.9±3.2	35.9±3.1	36.6±1.8	36.8±4.2
Immunox 3-6	38.5±2.2*	39.9±3.3*	43.4±4.1*	45.3±3.1*	46.2±4.1*	46.9±3.2*
Betacan 500	32.1±1.8	33.2±2.8	34.8±3.3	36.6±2.8	35.8±2.9	37.7±3.2
Glucan Real	31.8±1.8	34.2±3.3	37.1±1.2*	40.2±1.7*	42.2±1.9*	44.4±2.9*
MC-Glucan	31.8±1.6	34.1±0.9	34.1±2.7	38.1±1.9	38.9±2.2	40.7±2.2
Beta Glucan (Germany)	32.6±2.2	35.1±2.1	37.1±1.8*	37.9±2.2*	38.1±1.9*	41.1±2.1*
Barley Glucan	31.8±1.1	32.1±0.8	33.1±0.9	34.1±2.1	35.8±3.2	34.8±2.2
Beta Glucan (Vitabase)	32.6±2.2	32.6±0.8	33.1±1.9	32.9±2.3	34.7±2.8	35.1±4.3
Reishi	31.7±0.7	33.1±0.9	35.6±1.9	37.6±1.0*	38.9±2.4*	40.6±2.7*
Beta Glucan (Greenpath)	30.9±0.9	31.8±1.1	33.1±1.5	34.0±1.1	34.6±2.3	35.1±3.3
Hliva ustricna	31.5±1.1	32.8±2.1	34.1±2.4	35.2±3.0	34.7±2.4	35.1±2.8
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 31.3±2.7. The dose means a single ip. injection in PBS/mouse.
*Significant differences between glucan and PBS at <0.05 level. Results shown as percentage of phagocytosing blood neutrophils represent mean±SD, n was always more than 10.

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demonstrate the effects of various doses of tested glucan on phagocytosis of peripheral blood neutrophils. Several trends can be observed–clear dose-dependency, several glucans showed no activity even at the highest doses, and the most active glucan (Glucan #300) reached the plateau at a dose of 100 µg, with the level of stimulation not achieved by other glucans even at a dose of 800 µg. The glucans with consistent significant effects were Immunox 3-6 and Glucan #300. Several others were active from the higher doses (Bio-Glucan, Glucan-Real, Reishi and Beta Glucan from Germany).

Phagocytosis results in internalization of the prey, but represents only one of the several subsequent steps, leading to burst of metabolic activity and final killing and/or destruction of the ingested material. Therefore, we evaluated the effects of our glucans on production of superoxide anion. To make sure the test produced accurate data, we used two experimental *in vitro* models–human cell line HL-60 and mouse neutrophils. Data shown in **Table 3** confirmed that almost all tested glucans significantly increased the formation of superoxide anion, with only Oat Beta Glucan and Barley Glucan having no activity at all. The most active glucan was Glucan #300 followed by Bio-Glucan and MC-Glucan. For comparison, the levels obtained using resveratrol-vitamin C-glucan mixture reached 1.99 nmol/2.5x10^5 cells.

Glucans also have significant effects on various cytokines. To compare the effects of our group of glucans, we measured the production of IFN-γ in the blood (*in vivo* experiment) and IL-2 by splenocytes (*in vitro*). The secretion of IL-2 by untreated murine splenocytes is zero, therefore all glucans significantly increased the IL-2 production (**Table 4**). It is clear, that the Concanavalin A elicited the highest response, with Glucan #300 being close. Several other glucans showed high activity–Bio-Glucan, Immunox 3-6, Glucan Real and MC-Glucan. Similar effects were seen in stimulation of IFN-γ secretion. Again, due to absolutely minimal level of IFN-γ in control mice, all glucan caused statistically significant stimulation. The glucans with highest activity were Glucan #300, Immunox 3-6, Beta Glucan (Germany) and Reishi. As positive control, we used *in vivo* stimulation with LPS which increased the IFN-γ level in the blood up to 400-500 pg/ml.

Table 3. Effect of individual glucans on superoxide anion production.

Glucan	Mouse neutrophils (nmol/2.5x10^5 cells)	HL-60
BetaMax	1.12±0.11*	1.23±0.25*
Oat Beta Glucan	0.35±0.05	0.44±0.11
Bio-Glucan	1.44±0.23*	1.48±0.37*
Qore Defense	0.65±0.24*	0.64±0.15*
Immunox 3-6	1.07±0.25*	1.22±0.21*
Betacan 500	0.87±0.30*	0.79±0.29*
Glucan Real	1.31±0.25*	1.43±0.36*
MC-Glucan	1.44±0.41*	1.55±0.26*
Beta Glucan (Germany)	0.78±0.22*	0.99±0.32*
Barley Glucan	0.38±0.09	0.43±0.12
Beta Glucan (Vitabase)	0.78±0.13*	0.88±0.23*
Reishi	0.99±0.23*	1.12±0.34*
Beta Glucan (Greenpath)	0.76±0.22*	0.89±0.24*
Hliva ustricna	0.56±0.12*	0.75±0.21*
Glucan #300	1.69±0.34*	1.55±0.27*
PBS	0.25±0.08	0.35±0.07

*Significant differences between glucan sample and PBS control at P<0.05 level. Results represent mean±SD, n was always more than 10.

Table 4. Effect of individual glucans on IL-2 and IFN-γ secretion.

Glucan	IL-2 (pg/ml)	IFNγ (pg/ml)
BetaMax	78.3±8.9	25.3±1.9
Oat Beta Glucan	62.2±5.5	41.2±2.5
Bio-Glucan	363.3±14.4	65.2±4.0
Qore Defense	30.1±2.1	30.3±2.4
Immunox 3-6	611.1±83.9	116.1±7.8
Betacan 500	87.9±6.6	27.9±2.1
Glucan Real	442.2±87.5	82.3±2.5
MC-Glucan	459.9±64.4	59.9±2.4
Beta Glucan (Germany)	223.6±11.8	103.6±5.8
Barley Glucan	12.9±1.1	22.3±1.0
Beta Glucan (Vitabase)	230.8±11.3	39.8±1.1
Reishi	288.8±24.4	128.5±4.9
Beta Glucan (Greenpath)	174.4±36.6	84.0±6.2
Hliva ustricna	39.9±3.2	9.9±0.8
Glucan #300	983.9±122.8	201.2±11.5
Con A	1 103.3±291.2	ND
PBS	0	2.1±0.2

All glucans showed significant stimulation of IL-2 secretion at P<0.01 level. The PBS control showed no IL-2 production. All glucans showed significant stimulation of IFNγ secretion when compared to PBS (P<0.01 level). Results represent mean±SD, n was always more than 10.

In the next step, we focused on the role of tested substances in cancer development. As an experimental model, we used mice challenged with Ptas64 mammary tumors. Two weeks of glucan injections caused significant reduction of cancer growth (measured as tumor weight) in five cases–Glucan #300, Immunox 3-6, Glucan Real, Beta Glucan (Germany) and Reishi. In all other cases, the reduction was either statistically insignificant or the glucans had no effects at all (**Table 5**).

In the last part of our study, we evaluated the less known area of glucan effects-antibody response. We used an immunization of mice with ovalbumin, where glucans were applied

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together with two separate intraperitoneal injections of antigen. As positive control, ovalbumin was used with Freund's adjuvant. The results summarized in **Table 6** showed that six different glucans significantly increased the specific antibody response–Qore Defense, Immunox 3-6, Glucan Real, Beta Glucan (Germany), Reishi and Glucan #300/.

Table 5. Effect of individual glucans on suppression of breast cancer.

Glucan	Tumor weight (mg)
BetaMax	512.7±49.9
Oat Beta Glucan	501.7±45.5
Bio-Glucan	499.1±46.2
Qore Defense	501.3±33.7
Immunox 3-6	348.9±40.2*
Betacan 500	522.3±47.8
Glucan Real	467.7±34.7*
MC-Glucan	511.8±40.1
Beta Glucan (Germany)	476.2±38.8*
Barley Glucan	611.6±53.6
Beta Glucan (Vitabase)	512.8±42.4
Reishi	411.1±32.7*
Beta Glucan (Greenpath)	601.0±52,3
Hliva ustricna	603.5±55.6
Glucan #300	286.1±23.5*
PBS	622.6±52.5

*Significant reduction of tumor weight at P<0.05 level (individual glucans vs. PBS control). Each group consisted of at least 9 mice evaluated in three independent experiments. Results represent mean±SD.

Table 6. Effect of individual glucans on antibody formation.

Glucan	% of control
BetaMax	102.4±10.8
Oat Beta Glucan	111.6±21.2
Bio-Glucan	125.4±14.8
Qore Defense	133.1±12.5*
Immunox 3-6	296.1±17.2*
Betacan 500	128.3±20.3
Glucan Real	201.3±18.5*
MC-Glucan	129.9±9.9
Beta Glucan (Germany)	207.6±16.8*
Barley Glucan	111.9±11.0
Beta Glucan	130.6±14.8
Reishi	189.8±14.7*
Beta Glucan	126.1±17.2
Hliva ustricna	109.2±9.8
Glucan #300	343.9±43.1*
Ovalbumin+adjuvant	509.9±45.5*

*Significant stimulation at P<0.05 level. Results represent mean±SD, total number of mice was 9/group.

Discussion

Glucans are carbohydrates consisting of linked glucose molecules, which are major structural components of the cell walls of yeast, fungi and some bacteria. In addition, cereals such as barley and oat contain glucans as a part of their endosperm. Glucans are the most studied natural immunomodulators which, due to the numerous ongoing human clinical trials, have the strongest chance to become an approved drug even in Western medicine. However, it is often difficult to compare the effects of glucan differing in source, isolation techniques, solubility and other physicochemical characteristics such as branching or molecular weight. These comparisons are possible only when individual glucans are compared in one study using identical experimental design. Despite thousands of scientific papers, often describing new and new types of glucan, comprehensive reviews comparing individual biological or immunological activities are rare. Most of them are focused more on the relation between biological activities and chemical properties [21,22], which does not fully help to answer the question which glucan is better. Other comparative studies focused on comparison of glucans extracted from oat, wheat or barley, but the studied effects were focused on effects on liver and glucose regulation [15]. However, there are no similar comparative studies on glucan and immune reactions.

In our previous work, we directly compared 16 different glucans [16]. From the time of publishing of the original study, the number of commercially available glucans multiplied in numerous countries. This inspired us to compare the new batch of available glucans. In the present paper, we used some of the same reactions (phagocytosis, superoxide formation, antibody reaction and IL-2 secretion) that have already been published. However, the original study showed that some glucans stimulate some types of immune reactions, and are without any activity in other areas of immunity. Therefore, for better evaluation of individual glucans, we added two more activities - IFN-γ secretion in blood and suppression of breast cancer growth.

Phagocytosis usually represents the first studied effects of glucan, as this molecule was originally described as nonspecific modulator of macrophages. In our study, we employed the synthetic microbeads based on 2-hydroxyethyl methacrylate polymer, since they represent good experimental material for these types of the study. These microbeads are known for their minimal nonspecific adhesion to the cell membrane, thus limiting the false positivity [20]. Our data showed that 50% of the tested glucans had no stimulative activity even after the highest dose (800 μg). On the other hand, the best glucans demonstrated significant activity even at the lowest dose. The differences in dose required to elicit significant stimulation might be up to 8x. In addition, most glucans did not reached the activities of the most active glucan even at 32x higher dose.

Another part of the internalization process is the subsequent burst of metabolic activity. Part of it is the production

of active oxidative species, necessary for killing and destruction of bacteria (for review see [23]). Glucans were repeatedly shown to stimulate oxidative burst [24,25]. All mushroom-and yeast-derived glucans stimulated production of superoxide anion, where as oat-derived glucans did not. One can only speculate why the oat glucan had no such activity, even when they can be as active in cancer inhibition as glucan from other sources. The most probable explanation might be the low purity of the oat glucans used in this study or by higher viscosity of these glucans.

Glucans are well known to stimulate production and secretion of various cytokines, with a wide range from IL-1, IL-2, and IL-6 to TNFα, and IFNγ [26,27]. In fact, there is only one known glucan without any significant stimulation of cytokine production [28]. For our purposes, we measured the effects of glucans on production of IL-2 by splenocytes and level of IFNγ in peripheral blood. Under normal circumstations, splenocytes do not produce IL-2, so the basal levels are almost zero. As a result, all glucans showed significant stimulation of Il-2 production, with Glucan #300, Bio-Glucan, Glucan Real, Immunox 3-6 and MC-Glucan showing highest effects. However, only Glucan #300 reached levels comparable with positive control (Concanavalin A). A similar situation has been found in case of IFNγ, where the strongest activity was associated with Glucan #300, Immunox 3-6, Beta Glucan (Germany) and Reishi. It is clear, therefore, that individual glucans significantly differ in their abilities to stimulate production and/or secretion of individual cytokines.

Recently, glucans have been shown to stimulate not only the cellular branch of immune reactions, but also the antibody formation [29,30], leading to suggestions that glucan can be part of vaccination. In farmed animals such as fish or chicken, glucan inclusion in vaccine is already being intensively studied [31,32]. Six of our group of glucans significantly stimulated secretion of specific anti-ovalbumin antibodies, with Glucan #300 being the most active one.

The last part of our study was devoted to the effects of glucans on breast cancer growth. We used previously established technique using murine cell line [33]. Five of our glucans significantly decreased the growth of breast cancer cells.

Conclusions

Our study clearly demonstrated that there are severe differences in immunological activities among our selected group of glucans. Similarly to our previous study [16], we tested fifteen varieties of glucans differing in source and solubility. Based on previous studies, we included Glucan #300 as the benchmark. We confirmed that some glucans can have significant effect on some defense reactions, whereas have little or no activity on others (e.g., Qore Defence had no activity on tumor suppression, but stimulated antibody secretion). Several glucans consistently showed higher biological activities, most of all Immunox 3-6, Glucan Real, Beta-Glucan (Germany) or Reishi, but in every tested reaction, the Glucan #300 was the most active sample. The differences between individual glucans found in this report might explain the sometimes confusing results published in the literature. It is clear that the immunological and biological effects of individual glucan are not connected to their source or solubility.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	VV	JV
Research concept and design	✓	✓
Collection and/or assembly of data	✓	✓
Data analysis and interpretation	✓	✓
Writing the article	✓	✓
Critical revision of the article	✓	✓
Final approval of article	✓	✓
Statistical analysis	✓	✓

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