A Comparison of Injected and Orally Administered β-glucans

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ABSTRACT

β-glucans have been extensively studied for their pharmacological effects. Despite in-depth research, little is known about the optimal dose and/or optimal route of application. In this paper, we are reporting the result of comparing the immunostimulating activities of four commercially available glucans differing both in their solubility and source. In addition, we compared intraperitoneal and oral application, and the differences between a single versus repeated doses.

Our data showed strong differences in activities of individual glucans, with glucan yeast-derived #300 being the best, and grain-derived ImmuneFiber being the worst. Furthermore, we demonstrated that oral delivery of glucan resulted in significant immunological activity, which albeit slightly lower, corresponded with injectable application. Depending on the applied dose, the effects of individual glucans were long-lasting and in some cases, lasted up to two weeks.

In conclusion, our report represents further evidence about differences among commercial glucans and shows that these biological response modifiers can be similarly active when used in both injectable and oral form.

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INTRODUCTION

Natural products, useful in treating or preventing various diseases, have been sought throughout the history of mankind. Most of these natural products are plagued with a common problem, i.e., the fact that they often represent a complex mixture of individual ingredients, each of which can contribute to their biological activities. Natural (1,3)-β-D-glucans from yeast, grain and mushrooms are well-established biological response modifiers,[1,2] representing highly conserved structural components of cell walls in yeast, fungi, seaweed, or grain seeds.

Numerous types of glucans have been isolated from almost every species of yeast, grain and fungi. (1,3)- β-D-glucans have been extensively studied for their immunological and pharmacological effects. More than 2,000 papers describing the biological activities of glucans exist in the literature.[3] Another advantage of glucans is the fact that all sufficiently purified polysaccharidic immunomodulators distinguish themselves by very low toxicity (*e.g.*, for mouse lentinan has $LD_{50} > 1600$ mg/kg[4]).

Despite detailed knowledge of the activities of many glucans, limited information is available regarding the mechanisms of action by orally delivered glucans. For some time, there were even suggestions that orally administered glucans have no activity at all. Only recently has more information about the mechanisms of action of orally delivered glucans become available.[5,6]

The limited number of papers dealing with the problems of glucan transfer through the gastrointestinal tract mainly focus on the fact that fluorescent-labeled glucan can be detected in cells isolated from various tissues.[7] The studies of Ross's group indicated that orally-administered (1,3)-β-D-glucan is taken up by gastrointestinal macrophages

and subsequently shuttled to the reticuloendothelial system and bone marrow. Recent observation found that both insoluble glucans and soluble seaweed-derived Phycarine have similarly pronounced effects when applied via intraperitoneal or oral administration.[7,8,9]

The aims of the present study were to follow up our previously published comparison of commercial β-glucans[10] and to test the effect of different commercially available glucans on both the cellular and humoral branches of immune reactions using different routes of administration.

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_2 asphyxiation.

Materials

RPMI 1640 medium, sodium citrate, dextran, ovalbumin, Ficoll-Hypaque, antibiotics, sodium azide, bovine serum albumin, Wright stain, Limulus lysate test E-TOX-ATE, 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).

β -1,3 glucans

The glucans used in this study were purchased from the following companies: NOW BETA glucan from NOW FOODS (Bloomingdale, IL), Krestin from Biotec (Kureha Chemical Industries, Tokyo, Japan), Glucan #300 from Transfer Point (Columbia, SC), and ImmunoFiber from (Whole Control, Arvada, CO).

Glucan treatment

Individual glucans were applied either intraperitoneally or orally. The samples were collected at different intervals after either single or three ip. injections (100 mg of glucan/mouse) or after one day or a fourteen-day feeding with glucan-containing diet. All diets (Laboratory Rodent Diet 5001 enhanced with various doses of glucan) were formulated and prepared by Purina (Richmond, IN). Diet ingredients for all groups were identical except for the proportion of glucan.

Antibodies

For fluorescence staining, the following antibodies have been employed: anti-mouse CD4, CD8 and CD19, conjugated with FITC, which 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 $5x10^5$ cells were incubated with 10 µl of FITC-labeled antibodies (1 to 20 µg/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 that employs phagocytosis of synthetic polymeric microspheres was described earlier.[11,12] Briefly: peritoneal cells were incubated 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. The same smears were also used for evaluation of cell types.

Evaluation of IL-2 production

Purified spleen cells ($2x10^6$/ml in RPMI 1640 medium with 5% FCS) were added into wells of a 24-well tissue culture plate. After the 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_2). At the endpoint of incubation, supernatants were collected, filtered through 0.45 mm filters and tested for the presence of IL-2. Levels of the IL-2 were measured using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

RESULTS

Most published studies describe effects of injected β-glucans (either ip., iv. or sc.). However, it is necessary, in the event of clinical practice, to evaluate the possibility of oral delivery.

Phagocytosis is one of the biological activities traditionally connected with effects of immunomodulators, including glucans. Therefore, we started our study by comparing the effects of orally and intraperitoneally applied glucans. When used as a single dose, ip. application showed more profound effects than oral application (Figure 1 A,B). In addition, some glucans (such as NOW and Krestin) exhibited either longer effects or were effective only after injection. When we repeated the glucan administration for three consecutive days, we found not only higher phagocytic activity, but that it also lasted significantly longer (in the case of #300 and Krestin, up to 7 days). Oral delivery also showed higher effects, but similar to a single dose, signifi-
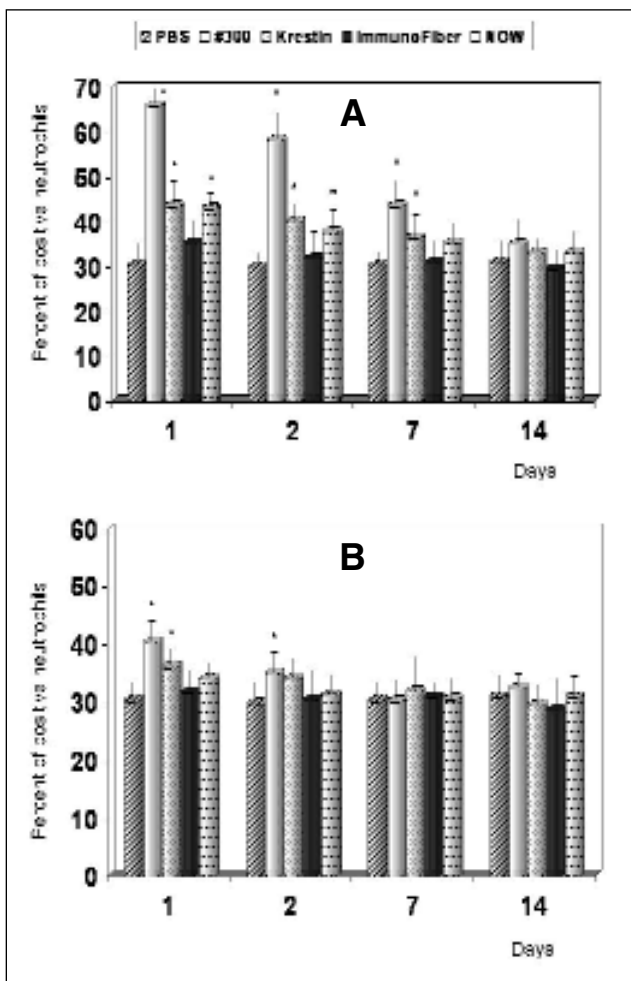
Figure 1.
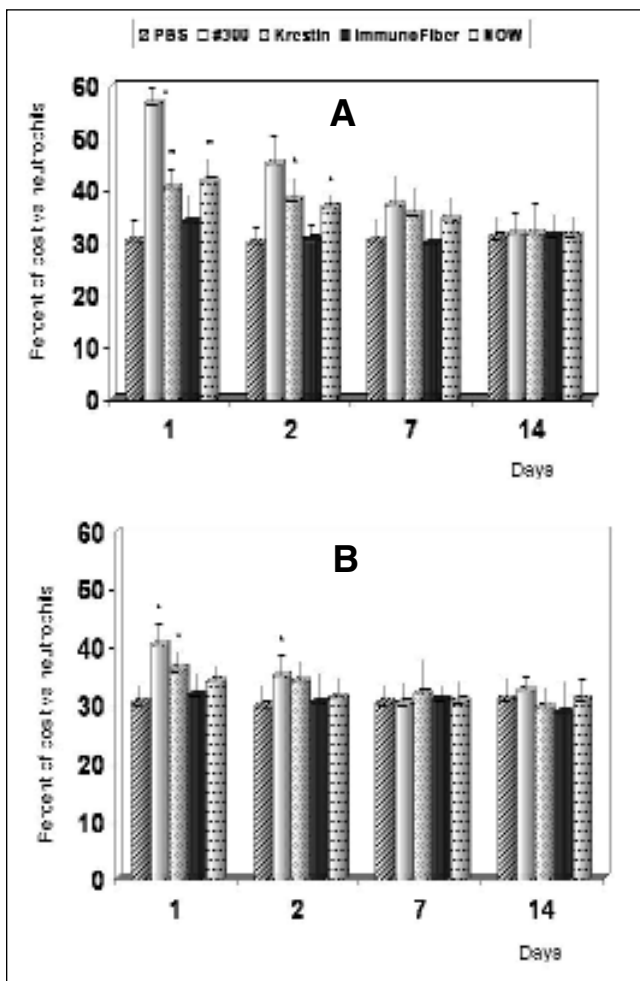
*Effect of an administration of 100 μg of different glucan samples on phagocytosis by peripheral blood granulocytes (A intraperitoneally, B orally). Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.*

Figure 2.

*Effect of an administration of 100 μg of different glucan samples on phagocytosis by peripheral blood granulocytes (A intraperitoneally, B orally). Glucans were applied three times. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.*

cant effects were observed only in the case of #300 (Figure 2 A,B).

Next, we evaluated the effects of our glucans on the expression of some immunologically important surface markers on spleen lymphocytes isolated from mice stimulated with individual glucans. Using ip. injection, we found that 24 hrs. later, all glucans increased expression of CD4, but this effect was long-lasting only in the case of #300 (Figure 3A). When testing CD8 expression, the effects of three active glucans, #300, NOW and Krestin, were observed for 48 hrs. (Figure 4 A). None of the tested glucans affected the number of CD19-positive cells (B lymphocytes (Figure 5A).

A similar situation has been found in orally-stimulated mice; the only exception was no activity of ImmunoFiber (Figures 3B,4B). Again, no effects on expression of CD19 (Figure 5B).

Production of IL-2 belongs to the valuable indicators of the immune activities. Therefore, we compared the effects of tested glucans on the secretion of IL-2 by spleen cells isolated from glucan-treated mice. The IL-2 production was measured after a 72 hr. *in vitro* incubation of cells. The results, summarized in Figure 6, showed that even when all tested glucan stimulated IL-2 production, there were huge differences between individual glucans (i.e., #300 stimulated IL-2 secretion 3.5 times more than ImmunoFiber). The activity of all tested glucans slowly decreased with time, but was still measurable 14 days after injection (Figure 6A). Virtually identical, albeit lower, results were found in the case of orally-treated mice (Figure 6B).

When we evaluated the IL-2 production after repeated stimulation with glucans, we found a higher overall secretion of IL-2. Using ip. injection, #300 was more active than
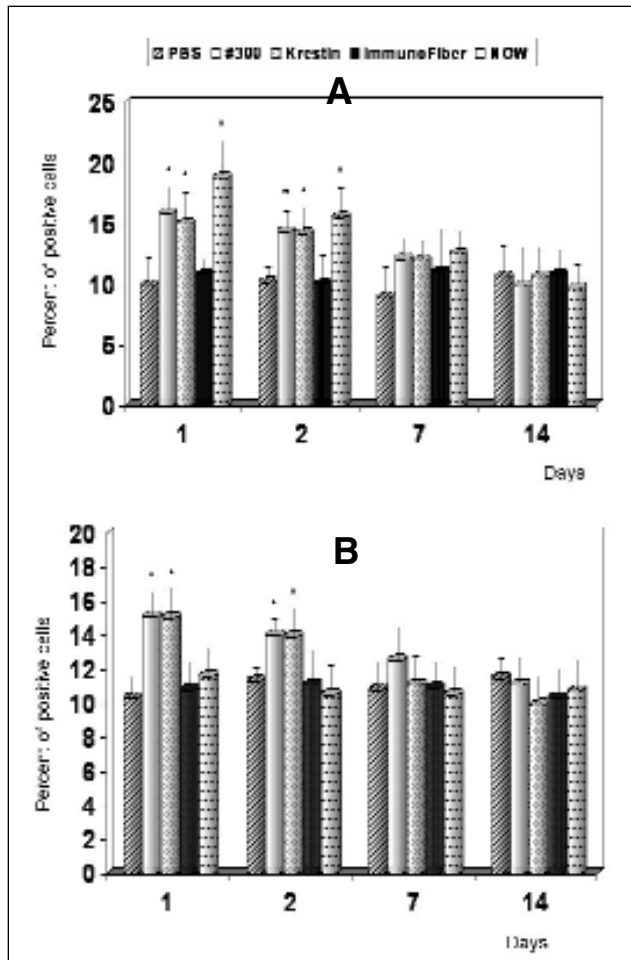
Figure 3.
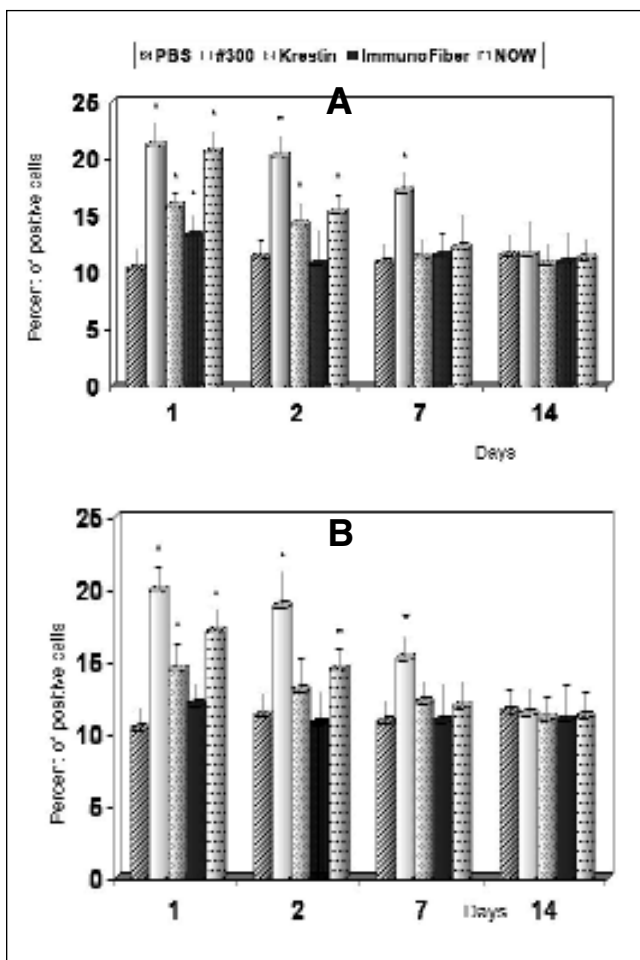
Effect of application of 100 μg of tested glucans on the expression of CD4 marker by spleen cells (A intraperitoneally, B orally). The cells from three donors at each time interval were examined and the results given represent the means ± SD.

*Represents significant differences between control (PBS) and samples at P ≤ 0.05 level.

Figure 4.

Effect of application of 100 μg of tested glucans on the expression of CD8 marker by spleen cells (A intraperitoneally, B orally). 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.

Concanavalin A up to seven days after last application, with both Krestin and NOW showing strong stimulation (Figure 7A). The same situation was found after oral application, where we discovered significant stimulation in each glucan even two weeks after the last application (Figure 7B). It is important to note that the secretion of IL-2 by control (i.e., non-stimulated cells) was almost zero; therefore, all glucans yielded statistically significant stimulations. When compared to stimulation with Con A, #300 showed stronger effects, Krestin and NOW were comparable, and ImmunoFiber showed lower activity.

Glucans are usually considered more as stimulators of the cellular branch of immune reactions; however, some glucans can act as nonspecific adjuvant. Using an experimental model of ovalbumin immunization, we applied glu-

can either intraperitoneally together with two doses of antigen (Figure 8A), or orally for two weeks (Figure 8B). In both cases, only glucans #300, Krestin and ImmunoFiber showed stimulation of antibody response.

Finally, we evaluated whether the glucan feeding was reflected in changes of weight of individual organs. As seen in Table 1, there were no differences in the weight of any tested organs. In addition, the ip. injection had no effects (results not shown).

DISCUSSION

β-Glucans show notable physiological effects, which is the main reason why so much attention has been devoted to them. They belong to a group of physiologically active

Figure 5.
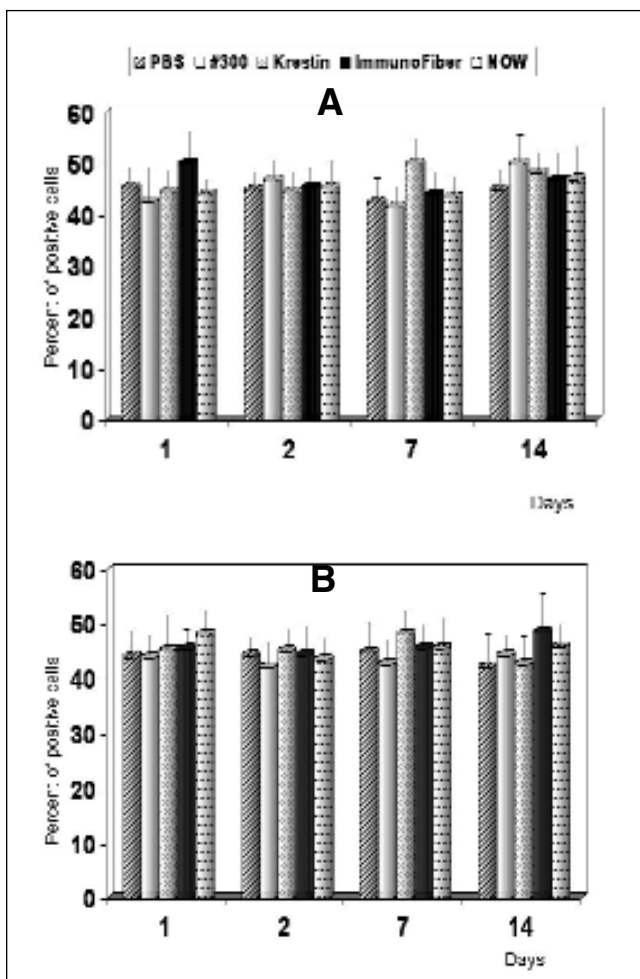
Effect of application of 100 ?g of tested glucans on the expression of CD19 marker by spleen cells (A intraperitoneally, B orally). The cells from three donors at each time interval were examined and the results given represent the means ± SD.
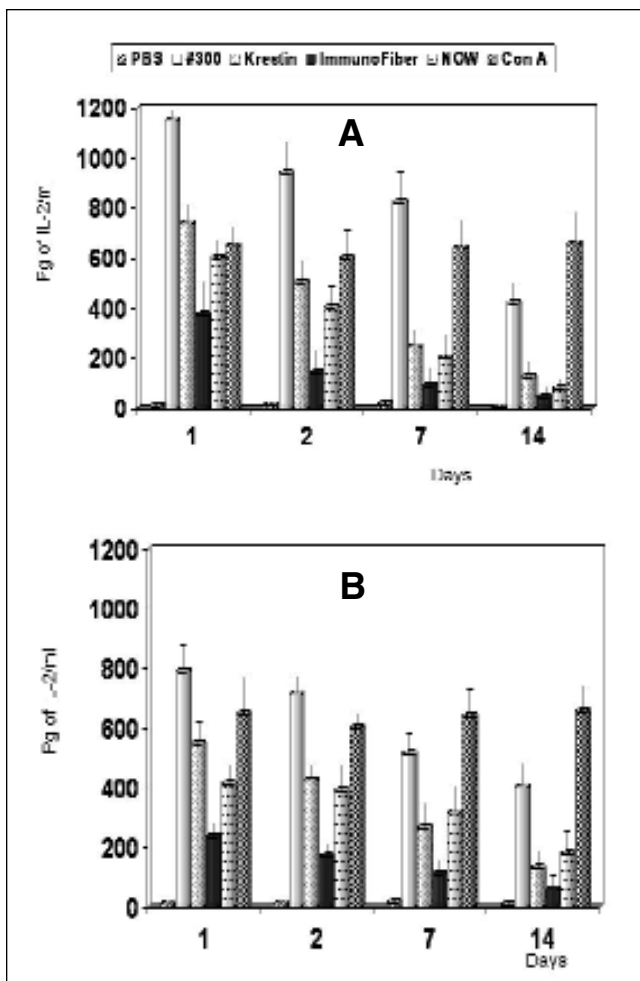
Figure 6.
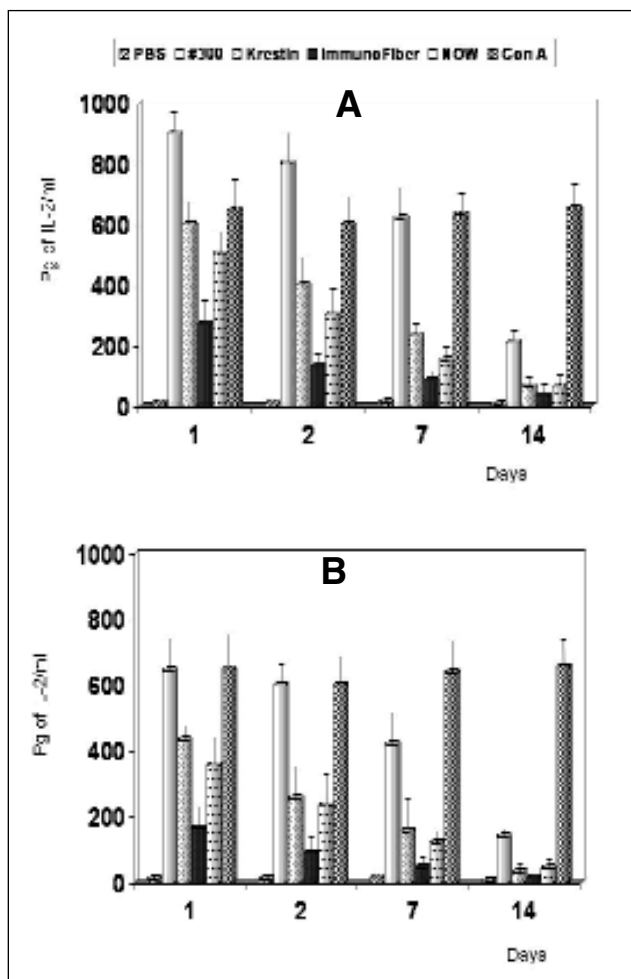
Effects of glucans on Con A-stimulated secretion of IL-2 by spleen cells (A intraperitoneally, B orally).

Table 1.

	Control	**#300**	**Krestin**	**ImmunoFibre**	**Now**
Total weight	23.33 ± 1.06	24.34 ± 1.27	24.11 ± 1.48	24.99 ± 4.11	26.99 ± 2.77
Liver	1.77 ± 0.23	1.49 ± 0.31	1.55 ± 0.19	1.54 ± 0.38	1.47 ± 0.35
Spleen	0.16 ± 0.09	0.17 ± 0.06	0.14 ± 0.05	0.17 ± 0.07	0.16 ± 0.03
Thymus	0.05 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.08 ± 0.02
Heart	0.16 ± 0.06	0.17 ± 0.10	0.18 ± 0.08	0.16 ± 0.03	0.17 ± 0.03
Kidneys	0.14 ± 0.10	0.38 ± 0.07	0.40 ± 0.12	0.40 ± 0.15	0.42 ± 0.05
Lung	0.16 ± 0.02	0.17 ± 0.02	0.15 ± 0.02	0.18 ± 0.03	0.18 ± 0.05

Figure 7.

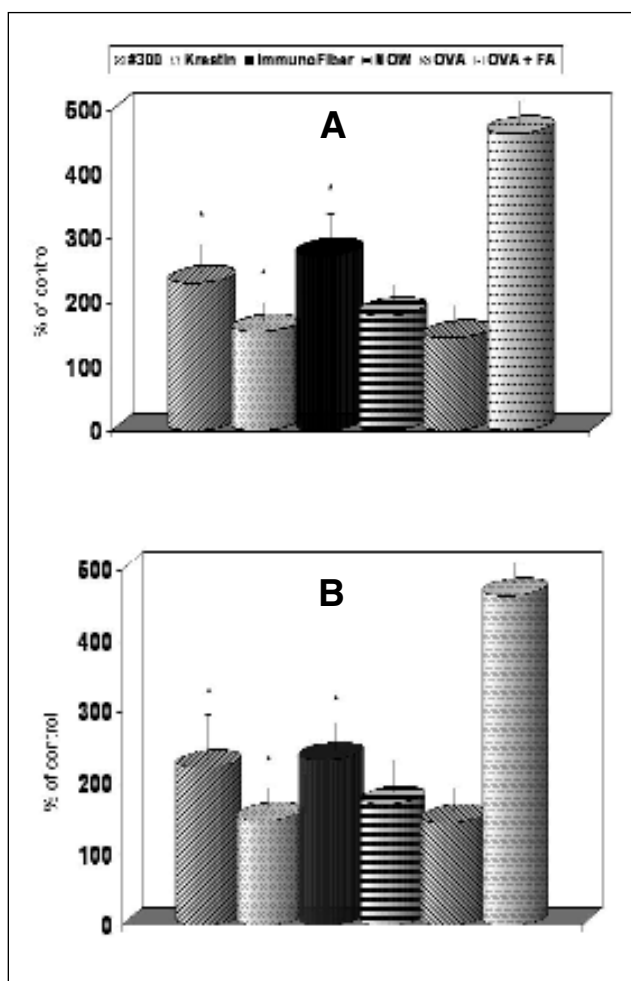*Effects of glucans on Con A–stimulated secretion of IL-2 by spleen cells (A intraperitoneally, B orally). Glucans were applied three times.*

Figure 8.

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

compounds, collectively termed biological response modifiers. Thus far, among many known and tested immunomodulators of the first order, polysaccharides isolated from different microorganisms and plants hold a formidable place. A large number of such polysaccharides, that act only as immunopotentiators are well known.[13]

Binding of β-glucan to specific receptors (either CR3 or Dectin-1) activates macrophages. The activation consists of several interconnected processes including increased chemokinesis, chemotaxis, migration of macrophages, degranulation leading to increased expression of adhesive molecules, and adhesion to the endothelium. In addition, β-glucan binding triggers intracellular processes, characterized by the respiratory burst after phagocytosis of invading cells (formation of reactive oxygen species and free radicals), the increase of content and activity of hydrolytic enzymes, and signaling processes leading to activation of other cells and secretion of cytokines. For an excellent

review regarding interaction of glucans with macrophages, see Schepetkin and Quinn.[14]

Regarding the question as to whether glucans are similarly active when administered orally, we compared the oral and intraperitoneal applications. To allow our experiments more relevancy in the use of natural immunostimulants, we compared the effects of a single application with repeated doses.

The rationale for the choice of glucans parallels what was stated in our previous paper.[10] We chose four glucans widely sold and available in the US, Europe, and the Far East, representing grain-, mushroom- and yeast-derived glucans in soluble and insoluble form. Briefly, #300 is insoluble yeast-derived glucan; Krestin is soluble mushroom-

derived glucan; ImmunoFiber represents soluble grain-derived glucan; and NOW is a mixture of both insoluble glucans from yeast and soluble glucans from mushrooms.

There are very few comprehensive reviews focused on biological properties of glucans from various existing sources. The comparative reviews focus mainly on the reflection of chemical characteristics of glucans on their biological and immunological properties.[15,16]

In this paper, we continued the comparison of several commercially important glucans.[10] Glucans are well known for their ability to stimulate the innate immunity and the cellular branch of immune reaction.[13] Therefore, our initial focus was phagocytic activity with the use of peripheral blood neutrophils and synthetic microspheres as a model. Our results confirmed our previous studies showing that glucan #300 was one of the most active glucans, regardless of the route of application.[8,10,17-19] Additional data showed that the duration of these effects depends on the strength and timing of the glucan treatment since repeated doses clearly resulted in stronger and longer action stimulation.

We then turned our attention to the effect of glucans on surface markers. In the case of CD4-positive lymphocytes, one injection of any of the glucans was enough to increase the influx of these cells. In the case of oral application, the data were similar with the exception of ImmunoFiber, which showed no activity. Similar data were observed in the case of CD8-positive splenocytes. In both cases, only #300, Krestin and NOW showed longer effects — two days for Krestin and NOW, and up to one week for glucan #300. The number of CD19-positive cells (B lymphocytes) did not change. These findings were in agreement with previous data established using Phycarine[20] or lentinan.[21] When we measured repeated doses of glucan, the results were identical to those shown in Figures 3 to 5, and due to the restricted space, were not included in this report.

It is assumed that glucan application results in signaling processes leading to activation of macrophages and other cells, and subsequent secretion of cytokines and other substances initiating inflammation reactions (*e.g.*, interleukins IL-1, IL-2, IL-6, and TNF-α).[22-24] We found that all tested glucans stimulated splenocytes to produce IL-2, with #300 and Krestin showing the strongest and longest effects. Our findings were similar to previously published data.[8,10,20]

As some recent studies established that glucans can also support the humoral branch of the immune reaction by serving as adjuvant,[25] we compared the adjuvant activities of tested glucans with Freund's adjuvant. Our results showed that even when the activities were always lower than those of Freund's adjuvant, they were nevertheless significant, with the higher activity found in the previously almost inactive ImmunoFiber. These data correlate well with the previous finding of significant adjuvant activity with grain-derived glucans.[10] It is important to note that in these experiments, we applied the glucans either two times ip. (together with the antigen) or for a full two weeks (in case of oral application).

The present paper represents yet another proof of vast differences among commercially available glucans. To conclude — glucan #300 was again a highly active glucan with a sufficiently broad range of action. We demonstrated that oral application is comparable to the intraperitoneal route, and that the somehow lower effects after oral stimulation can be easily overcome by repeated oral doses.

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