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# Immunological Effects of Yeast- and :Mushroom-Derived β-Glucans

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ABSTRACT Glucans have a long history as nonspecific biological modulators. We compared the effects of three different glucans on immune reactions. Using two different administrations (intraperitoneal and oral) and two different animal models, we showed that yeast-derived Betamune[n i] (Biorigin. Sao Paulo, Brazil) caused significant stimulation of phagocytic activity as well as potentiation of synthesis and release of interleukin (IL)- I. IL-2, IL-4, IL-6, IL-8. IL-13, and tumor necrosis factor-cr. In addition, Betamune inhibited growth of tumor cells ill *vivo* and affected expression of several important genes in breast cancer cells. Compared to adult mice, young animals showed different sensitivity to glucan action.

*KEY \VORDS:* • *gene* • *glucan* • *imm1111010gy* • *mushrooms* • *yeast*

## It TROOUCTION

β,3-Gu.;cA S ARESTRLCTUR-\LLY complex homopoly-

mers of glucose. usually isolated from yeast, fungi. wheat, and seaweed. B 1,3-Glucan·s role as a biologically active immunomodulator has been well documenred for over -W 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. t Further work identified the immunomodulatory active component as B 1,3-glucan.[1] Numerous studies (currently more than 2,000 publications) have subsequenrly shown that B 1.3-glucans, either particulate or soluble. exhibit immunostimulating properties, including anribacterial and antitumor activities.[3,4]

Despite decades of research on ,13-glucan, controversy still exists between the notion that water-insoluble glucans show only little bioactivity. whereas soluble glucans are highly active.[5] Numerous papers have shown that the question of bioactivity is more a question of purity and chemicophysical properties than solubility.[6,7] Various types of glucans have been isolated from almost every species of yeast and numerous fungi. This opens another unsolved question-which type of glucan has superior biological and/or immunological properties[1] Currently, no comprehensive review comparing effects of glucans isolated from different sources exists. In addition, there are probably more than a hundred different products on the U.S. market alone, leading to confusion about the quality, biological effects, and overall efficiency of glucan. In addition, some of the published reports describing individual glucans yielded rather confusing data. Therefore, we decided to evaluate the immunostimulating effects of a new yeast-derived glucan and compare it to with two well-established ,B-glucans: insoluble yeast-deri,ed whole glucan particle (WGP) and soluble mushroom-deri\·ed lentinan. In order to test the immunomoct1ilnrion e\·er further, we used two different deliveries--0ral and intraperitoneal. Young mice used in this srndy represent the first experiments regarding the effects of this substance on immarnre immune system.

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## MATERIALS ANO METHODS

### *Animals*

Female 3- (young) and 8- (adult) week-old BALB/c mice were purchased from Jackson Laboratory (Ba[r] Harbor, /'vfE). All animal work was done according to the University of Louisville (Louisville, KY) Institutional Animal Care and Use Committee protocol. Animals were sacrificed by COi asphyxiation. Each experimental group consisted of 15 animals.

### *t'vlaterials*

RPMl 1640 medium, sodium citrate, antibiotics, sodium azide. bovine serum albumin, ovalbumin, Wright stain, Freund's adjuvant, and concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum was from Hyclone Laboratories (Logan, UT).

### *Cell lines*

The human cell line ZR-75-1 was purchased from American Tissue Culture Collection (Manassas, VA). The

BALB/c mouse-derived mammary tumor cell line Ptas64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, Detroit, MI. The cells were maintained in RMPI 1640 medium supplemented with 10% fetal calf serum, 2 m*M* glutamine, and antibiotics.

### *β-1,3-Glucans*

The glucans used in this study were purchased from the following companies: yeast-derived WGP glucan was purchased from Biothera (Eagan, MN), yeast-derived Betamune™ glucan was obtained from Biorigin (São Paulo, Brazil), and the soluble mushroom-derived β-glucan, lentinan (molecular weight, approximately 1,000), was obtained from the Developmental Therapeutic Program, Division of Cancer Treatment, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

### *Phagocytosis*

The technique employing phagocytosis of synthetic polymeric microspheres was described previously.[8] In brief, peripheral blood cells were incubated with 0.05 mL of 2-hydroxyethyl methacrylate (HEMA) particles ($5 \times 10^8$/mL). The test tubes were incubated at 37°C for 60 minutes with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive.

### *Cytokine assay*

BALB/c mice were intraperitoneally injected with 100 μg of tested samples. Control mice received phosphate-buffered saline (PBS) only. After 24 hours, the mice were sacrificed, and blood was collected in Eppendorf tubes. Subsequently, the serum was prepared, collected, and stored at −80°C for no more than 1 week.

The levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α in serum samples were evaluated using commercial kits (OptEIA Mouse IL-1β or TNF-α Set, respectively; Pharmingen, San Diego, CA) according to the manufacturer's instructions. The optical density was determined using an STL enzyme-linked immunosorbent assay reader (Tecan U.S., Research Triangle Park, NC) at 450 nm with a correction at 570 nm. Data shown in Figure 3 were calculated from the standard curve prepared by the automated data reduction using linear regression analysis. A standard curve was run with each assay.

### *Cytokine array*

Human cytokines were measured in tissue culture supernatants by Allied Biotech (Ijamsville, MD). Tested cell lines were incubated for various time intervals with individual glucans in serum-free conditions. The supernatant was collected, filtered (pore size, 0.22 μm), and stored in a −80°C freezer. For the cytokine analysis, we used protein microarray services provided by Allied Biotech. In brief, the services used a sandwich antibody-based protein detection multiplex assay. A streptavidin-Cy5 conjugate was used for assay detection. The assay was done in quadruplicate with positive and negative controls spotted on each microarray. The assay detects the following cytokines: IL-2, interferon (IFN)-γ, TNF-α, IL-8, IL-12 p70, IL-12 p40, IL-4, IL-6, IL-10, IL-5, IP-10, macrophage inflammatory protein (MIP)-1β, IL-13, and IL-1β.

### *Evaluation of IL-2 production*

Isolated spleen cells ($2 \times 10^6$/mL in RPMI 1640 medium with 5% fetal calf serum) were added into wells of a 24-well tissue culture plate. After addition of 1 μg of concanavalin A into positive-control wells, cells were incubated for 72 hours in a humidified incubator (37°C, 5% $CO_2$). At the end point of incubation, supernatants were collected, filtered (pore size, 0.45 μm), and tested for the presence of IL-2.[9] Levels of IL-2 were measured using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

### *Tumor inhibition* in vivo

Mice were injected directly into the mammary fat pads with $1 \times 10^6$ per mouse of Ptas64 cells in PBS. The experimental treatment was begun after palpable tumors were found (usually 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 or by daily feeding (for 7 or 14 days). After treatment, the mice were sacrificed, and tumors were removed and weighed.

### *RNA extraction and reverse transcriptase (RT)-polymerase chain reaction (PCR)*

Total RNA was extracted from control and treated cells using Trizol™ reagent (Life Technologies, Inc., Gaithersburg, MD). RNA quality and quantity were determined by ultraviolet spectrophotometry and agarose gel electrophoresis. Two hundred nanograms of total RNA was reverse-transcribed using SuperScript™ One-Step RT-PCR with Platinum Taq kit (Invitrogen Inc., Carlsbad, CA) using gene-specific primers with the following conditions: 30 minutes at 50°C followed by 2 minutes at 95°C and then 25 cycles of 30 seconds at 95°C, 45 seconds at 52°C for nuclear factor (NF)-κB2 and β-actin (68°C for BCL-2, 43°C for CDC42), and 3 minutes at 72°C for NF-κB2 (45 seconds for CDC42 and BCL-2, 90 seconds for β-actin). The PCR was completed by 7 minutes at 72°C. RT-PCR products were then separated on a 1.0% agarose gel, visualized under ultraviolet light, and photographed. β-Actin served as the internal control.

### *Statistics*

Student's *t* test was used to statistically analyze the data.

## RESULTS

The number of individual glucans is almost as great as the number of sources used for their isolation. The rationale for the combination of glucan samples used in this project was not only their commercial availability and success, but, most importantly, comparison of both soluble and insoluble glucans and also glucans from different sources, including yeasts and mushrooms. All three glucans were tested using all three different doses (50, 100, and 250 $\mu$g per mouse). However, WGP and lentinan were active in the highest dose only; therefore only Betamune data are shown in full range of doses.

$\beta$-glucans are generally considered to be potent stimulators of cellular immunity, with macrophages and neutrophils being the most important targets. Therefore, we began our evaluation of glucans by testing of phagocytosis. We used synthetic polymeric microspheres (HEMA) since their use, dose, and timing are already well established in glucan studies.[9–11] Figure 1A shows the effects of a single injection of glucan on phagocytosis of HEMA particles in peripheral blood monocytes and neutrophils. Betamune was found to be more active in lower doses than WGP or lentinan. When we tested the effects of glucans on the same reaction in young animals, all three doses of Betamune caused significantly higher phagocytosis in both neutrophils and monocytes, whereas WGP and lentinan required the highest dose (Fig. 1B). We also tested the effects of orally given glucans. Figure 1C shows that 7- and 14-day oral administration of Betamune elevated levels of phagocytosis in both monocytes and neutrophils, while WGP stimulated only neutrophils, and lentinan was not active at all.

Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by spleen cells (Fig. 2), measured after a 72-hour *in vitro* incubation of spleen cells isolated from control and glucan-treated mice. Again, treatment of mice with Betamune glucan showed the highest stimulation. As the secretion of IL-2 by nonstimulated splenocytes is practically zero, every dose of glucan caused statistically significant stimulation. Another way to compare the effect on IL-2 formation and/or secretion is a comparison with stimulation caused by concanavalin A. In this case, the largest dose of each glucan caused significantly higher stimulation (Fig. 2A). Almost identical results were found in the case of young mice (Fig.

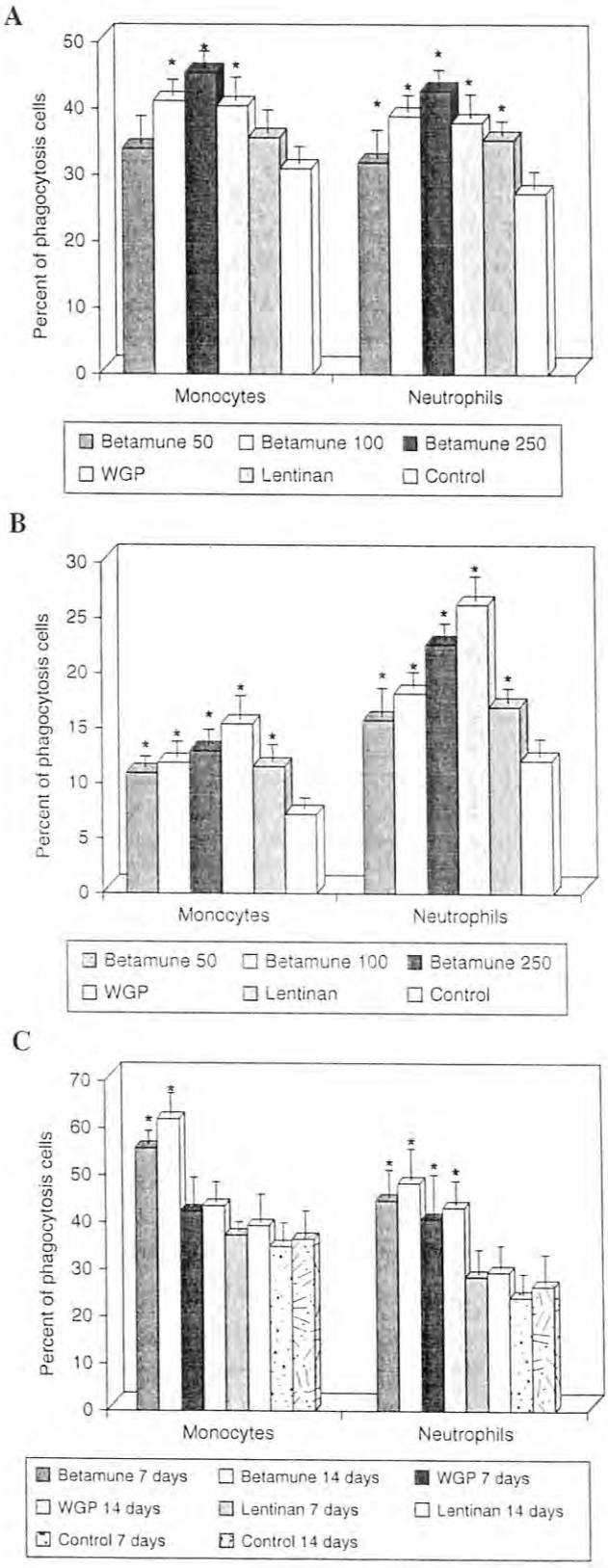


**FIG. 1.** (A) Effect of intraperitoneal administration of different glucan samples on phagocytosis by peripheral blood neutrophils and monocytes in adult mice. Betamune was used in three different concentrations (50, 100, or 250 $\mu$g per mouse); WGP and lentinan are shown at 250 $\mu$g per mouse only. Glucans were applied 24 hours before test. (B) Effect of intraperitoneal administration of different glucan samples on phagocytosis by peripheral blood neutrophils and monocytes in young mice. (C) Effect of orally given glucans on phagocytosis by peripheral blood neutrophils and monocytes in adult mice. Data are mean ± SD values. *Significant differences between control (PBS) and glucan samples at the $P \leq .05$ level.

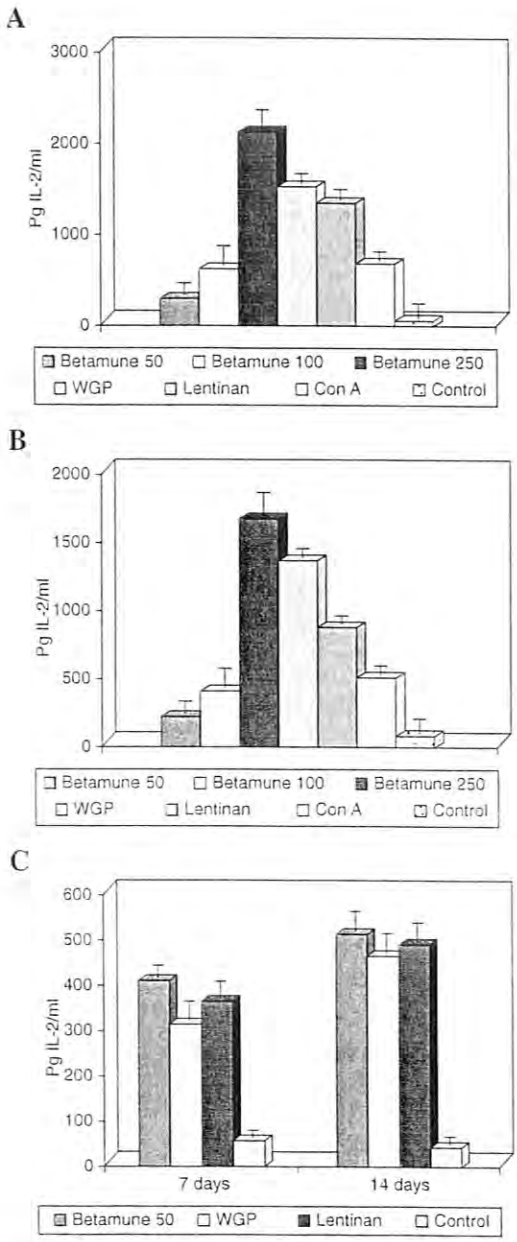


**FIG. 2.** Effects of *in vivo* glucan exposure on IL-2 secretion by cultured spleen cells: (**A**) adult mice, (**B**) young mice, and (**C**) oral treatment in adult mice. Data are mean ± SD values. The control group was fed with PBS. Con A, concanavalin A.

2B). When we tested the effects of oral feeding, all three glucans showed similar ability to potentiate IL-2 production (Fig. 2C).

We then compared the effects of a single intraperitoneal injection of the three different glucans on systemic *in vivo* release of IL-1$\beta$ and TNF-$\alpha$. Peripheral blood was isolated 24 hours after glucan injection, and the serum obtained was stored at −80°C for no more than 1 week. The data summarized in Figure 3 show that in adult mice both Betamune and lentinan caused significant stimulation of IL-1$\beta$ (Fig. 3A). Surprisingly, young mice showed a different response, with lower IL-1$\beta$ release and dramatically higher levels of TNF-$\alpha$, compared to adults. In this respect, WGP was the most active glucan (Fig. 3B).

The cytokine studies were further extended by experiments where we measured the secretion of cytokines from the cancer cell line ZR-75-1 24 hours after addition of glucan samples in serum-free conditions. Human cytokines were measured in tissue culture supernatants using protein microarray. Results given in Figure 4 show that out of the 14 cytokines tested (IL-2, IFN-$\gamma$, TNF-$\alpha$, IL-8, IL-12 p70, IL-12 p40, IL-4, IL-6, IL-10, IL-5, IP-10, MIP-1$\beta$, IL-13, and IL-1$\beta$), only four were detected. With the exception of IL-4, Betamune elicited the strongest cytokine response.

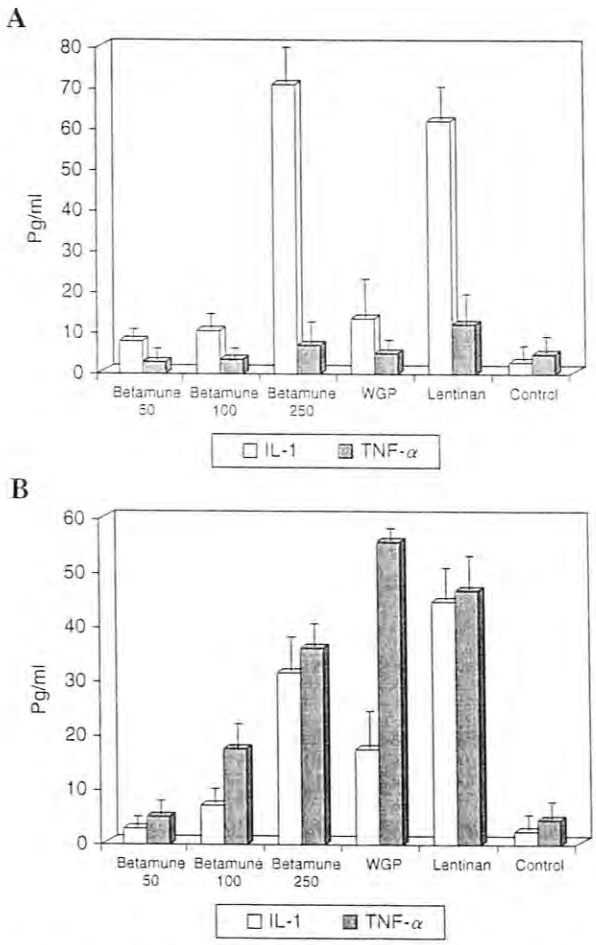


**FIG. 3.** Effect of intraperitoneal injection of glucans on levels of IL-1$\beta$ and TNF-$\alpha$ in peripheral blood: (**A**) adult mice and (**B**) young mice. Data are mean ± SD values. The control group was fed with PBS.

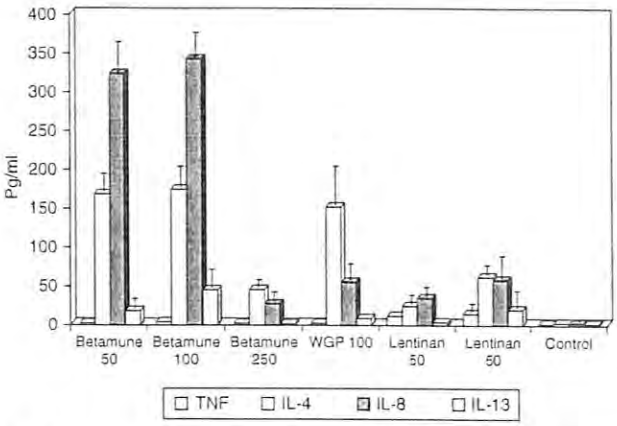


**FIG. 4.** Secreted cytokines 24 hours after addition of glucans to the ZR-75-1 cell line. Units of data on the y-axis are pg/mL of the respective cytokine. Data are mean ± SD values of three independent experiments. Other cytokines (as described in Materials and Methods) have not been detected. The control group was fed with PBS.

In the following experiments, mice challenged with Ptas64 mammary tumors were tested for a therapeutic response to daily intraperitoneal injections of individual glucans (Fig. 5A). Using orally administered glucans, we found almost identical data (Fig. 5B). This experiment was repeated three times with similar results and then was repeated with lipopolysaccharide-free glucans (data not shown). These data showed the strong inhibition of tumor growth by all three glucans. In the case of Betamune, the decrease of tumor weight was significant even at lower concentrations.

The RT-PCR given in Figure 6 shows the effect of the glucans tested on expression level of the genes *NF-κB2*, *cdc42*, *protein kinase C* (PKC), and *Bcl-2* in ZR-75-1 cells. A distinct up-regulation in expression levels of *Bcl-2* was evident in response to all tested glucans. In other cases, Betamune was the only glucan up-regulating expression of *NF-κB2* and *cdc42*, whereas in the case of *PKC*, both WGP and Betamune were active.

## DISCUSSION

High numbers of individual glucans have been described in the literature. Because of the huge differences in activities among various glucans isolated from numerous sources, it is imperative to evaluate its biological properties before any suggestions for use of a particular glucan in clinical practice. This investigation focused on the biological activities of three different types of glucan. In this paper, we compared three different glucans—two yeast-derived glucans and mushroom-derived lentinan. Both WGP and lentinan are well described,[12,13] and as lower doses evidenced no significant activity, we showed only the highest dose throughout the entire study.

Various types of immunomodulators, glucans in particular, are known to stimulate phagocytosis.[14] Therefore, the evaluation of this basic type of immune reaction is important to determine the effectiveness of any biologically active immunomodulator. We tested peripheral blood leukocytes for changes in phagocytosis, using synthetic microspheres based on HEMA, with only a slight negative charge and therefore no nonspecific adhesion to the cell surface. This guarantees that only actively phagocytosing cells will internalize these inert particles.[15] In addition to the effect of a single glucan injection into adult mice, we also tested young mice and oral delivery (in adult animals). In all three cases, we found a significant increase of number of phagocytosing monocytes after the Betamune treatment. In young mice, all glucans were highly active, with unsoluble WGP showing the highest stimulation. A similar situation was found in the case of neutrophils. The differences between the sensitivity of young and adult mice can be explained by significantly different properties of phagocytic cells isolated from young mice.[16–18]

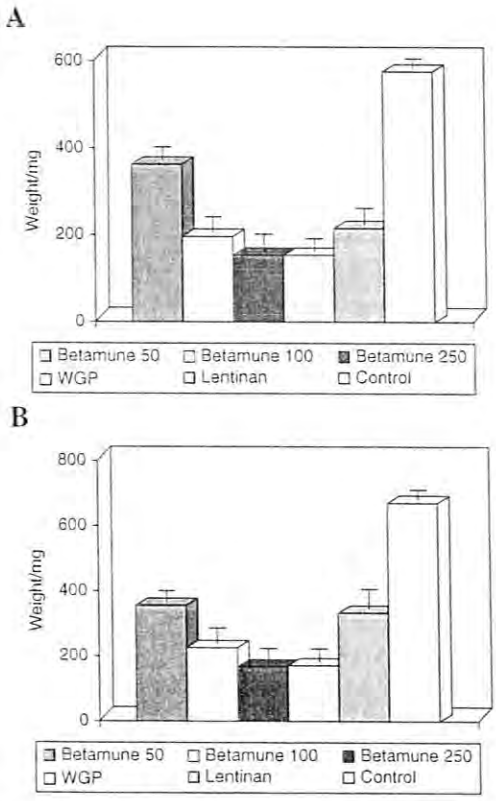


**FIG. 5.** Glucan therapy of BALB/c mice with Ptas64 mammary carcinoma. Data from four experiments are shown. For each experiment, groups of mice were tested for a response to glucans as indicated by the weight of tumors after 2 weeks of therapy. For each experiment, individual groups were given daily (**A**) intraperitoneal injections or (**B**) oral doses of 50, 100 or 250 μg of glucans, respectively. The control group of mice received daily PBS intraperitoneally. Data are mean ± SD values.

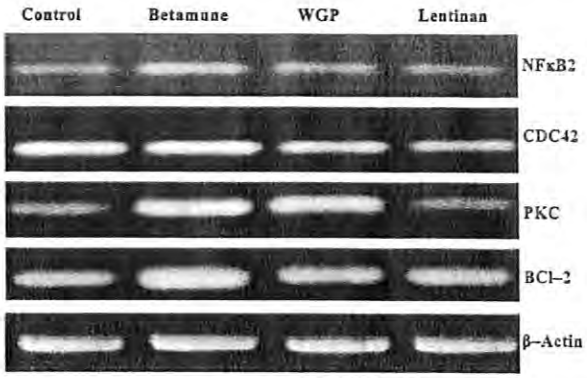


**FIG. 6.** Differential expression of the indicated genes in response to Betamune, WGP, or lentinan treatment. The control sample was treated with PBS. Equal amounts of RNA from control and treated ZR-75-1 cells were subjected to RT-PCR with gene-specific primers. As a control for equivalent loading, $\beta$-actin was expressed.

Currently, glucans are getting closer to use in clinical practice. In addition, orally effective lentinan is already being used in Japan.[19] It was therefore important to learn if these glucans can similarly improve the phagocytosis when used orally. We found that both 7- and 14-day oral administration of Betamune resulted in stimulation of phagocytosis of both monocytes and neutrophils, while WGP stimulated only neutrophils, and lentinan was not active at all.

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.[20] So far, the only known glucan without a trace of pro-inflammatory cytokine stimulation is PGG-glucan.[21] First, we focused on the stimulation of IL-2 production by spleen cells *in vitro* and found that all glucans increased the secretion, in both young and adult mice. Most glucans have been shown to stimulate TNF-$\alpha$ both *in vivo* and *in vitro*,[22,23] resulting in protective effects against infection.[24] When we focused on short-term stimulation, we surprisingly found that young mice responded with much higher production of TNF-$\alpha$ than adult animals.

The cytokine studies were further supported by measuring the effects of tested glucans on the breast cancer cell line ZR-75-1. Using a commercial array assay, we found that glucans stimulated only four (TNF-$\alpha$, IL-4, IL-8, and IL-13) cytokines. Similar data were found when we used human HaCaT keratinocytes (data not shown). These data resemble results showing molecular mechanisms of how the procathepsin D molecule exerts its effects on cancer cells.[25,26] It is, however, difficult to compare the stimulation of cytokine secretion by two completely different molecules such as glucan and procathepsin D.

Our previous studies demonstrated significant *in vitro* and *in vivo* inhibition of mouse and human breast tumor cell growth after $\beta$-glucan treatment.[10,27] Studies performed in numerous laboratories described antitumor activities of glucans in a series of tumor models, including hepatic carcinoma, sarcoma, and melanoma.[28] Our previous work demonstrated that there is a high similarity of mouse and human CR3 in response to glucans, which makes the mouse tumor models suitable for investigations of glucans.[27] We used the same experimental design and doses as published earlier, using various glucans such as yeast-derived[29] or seaweed-derived[10] glucans. All tested glucans had similar effects (approximately 60% inhibition of cancer growth), which were comparable to phycarine.[10] Our data showed strong inhibition of cancer growth *in vivo*, which can be caused by numerous factors, including effects on proliferation or apoptosis. However, out of more than 6,000 thousand publication, no report of glucan affecting proliferation or apoptosis *in vitro* exists.

Lipopolysaccharide contamination might mask the real effects of any glucan (or, more generally, any biological response modifier) preparation. Therefore, we checked the lipopolysaccharide contamination of Betamune solution and depleted any contamination by adding a 10 $\mu$g/mL solution of polymixin B. We then used this lipopolysaccharide-free Betamune in cancer inhibition experiments with identical results to those using regular Betamune. Therefore, the possible lipopolysaccharide contamination plays no role in the data described in this report.

Up-regulation of NF-$\kappa$B2 gene expression is considered significant as members of this family are important regulators of cell cycle progression, cell survival, cell adhesion/angiogenesis, invasion, and inflammatory responses.[30] They are also known to exert strong anti-apoptotic activity by inducing expression of several anti-apoptotic proteins like the Bcl-2 family proteins and interfering with expression or activity of pro-apoptotic proteins.[30] Studies have shown the positive role of NF-$\kappa$B family proteins in regulating the expression of adhesion molecules, matrix metalloproteinases, and angiogenic factors, which are known to increase the invasion and metastasis of cancer cells. CDC42 belongs to the RHO family of proteins and is implicated in the regulation of cell growth.[31] It has been reported that levels of CDC42 are elevated in many different human cancers, including human breast cancer,[32] and that they contribute to enhanced mitogenic signaling. In addition, another recent study has suggested that CDC42 can activate NF-$\kappa$B by a distinct pathway.[33] Of the glucans tested, Betamune showed the strongest effects, increasing the expression of all four genes tested, while WGP affected *cdc42* and *PKC*$\alpha$, whereas lentinan increased expression of *Bcl-2* only.

The recent isolation of a yeast-derived, highly purified $\beta$1,3-glucan with the tradename Betamune stimulated our interests in this immunomodulator. Our current paper clearly demonstrates that Betamune acts via the same mechanisms as other yeast-derived glucans and that it is,

in many cases, even more biologically active. When compared to lentinan, Betamune showed higher stimulation of defense parameters. These characteristics, together with the fact that it can be easily isolated in sufficient batches and high purity, make Betamune a prime candidate for commercial use.

## ACKNOWLEDGMENTS

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## AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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