Pilot Study:
Orally-Administered Yeast β1,3-glucan Prophylactically Protects Against Anthrax Infection and Cancer in Mice

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Yeast β1,3-glucan and Its Use Against Anthrax Infection and in the Treatment of Cancer

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In this edition of *JANA,* the paper by Vetvicka *et al*. makes an important contribution to our scientific understanding of the nutraceutical stimulation of the immune system in the treatment of both infectious disease and cancer. While abundant evidence demonstrates the ability of β1,3-glucans to activate macrophages and neutrophils when given intravenously or intraperitoneally, there has been little information concerning its efficiency when given orally.

In their study, Vetvicka *et al*. used oral β1,3-glucan (Imucell™ WGP Beta Glucan) from a yeast source in mice infected with *Bacillus anthracis*. With the high incidence of complications associated with anthrax vaccines, an alternative approach is badly needed in this era of bioterrorism threat. Dr. Ken Alibek, a top-ranking scientist at the Russian bioweapons labs, stated that because of the number of possible bioweapon agents available, something other than mass inoculations would be needed. He suggested non-specific immune stimulation. The most effective form of non-specific immune stimulation is macrophage activation.

The anthrax bacillus secretes two toxins, edema toxin and lethal toxin. Edema toxin stimulates an outpouring of fluid, especially into the lungs. Lethal toxin, inhibits neutrophil phagocytosis and triggers destructive intracellular reactions that destroy macrophage cells. Of primary interest is the fact that anthrax lethal toxin inhibits the macrophages from releasing their immune messengers, primarily IL-1, IL-2, IFN-gamma, and TNF-alpha.

Of particular importance in combating infection is the cytokine TNF-alpha. Vetvicka *et al.* demonstrated that yeast-derived β1,3 D-glucan given orally stimulates TNF-alpha release from the macrophage, apparently overcoming inhibition by anthrax lethal toxin. This would account for the high survival figures in the β1,3-glucan-treated animals. Some previous studies found no increase in TNF-alpha but a significant increase in IL-1β.[1] Other researchers have demonstrated increased TNF-alpha in response to ß-glucan stimulation.[2]

My own review of the literature confirms their statement that the most effective source of β1,3-glucan is from *Saccharomyces cerevisiae*, the one chosen by most researchers. Purity of the product is vital, since protein contaminants, as seen in the earlier-used source Zymosan, can cause untoward immune reactions.

β1,3-glucan also stimulates phagocytosis of neutrophils. In one study, the killing efficiency of neutrophils was increased 20- to 50-fold.[3] This is important since the capsular antigen poly-D-glutamic acid from the anthrax organism inhibits neutrophil phagocytosis. It is the two lethal toxins and the capsular antigen that makes the anthrax organism especially deadly. In addition, β1,3-glucan has been shown to increase clearance of bacteria by the reticuloendothelial system. Thus far, no other solutions have solved this problem.

As for β1,3-glucan's effects on tumor growth, several studies have shown a significant effect on tumor growth in animal models.[4,5] Early studies using immune stimulation found occasional tumor growth enhancement. This was later found to be secondary to stimulation of blocking antibody production. A safer and more effective method of immune stimulation is directed at cellular immunity, in particular the stimulation of T-helper cells and NK cells.

β1,3-glucan has been shown to increase lymphocyte production, NK cell activation, and activation of macrophages. Several studies have also demonstrated the role played by cytokines in inhibiting tumor growth; again, particular interest is in TNF-alpha release.[6] Of interest also is the role played by IL-1β, which is increased by β1,3-glucan as well. Interleukin 1β has been shown to enhance mobilization of PMLs in the bone marrow and enhance their chemotactic ability. In addition, IL-1β increases the lymphocyte count and increases their activity.[7]
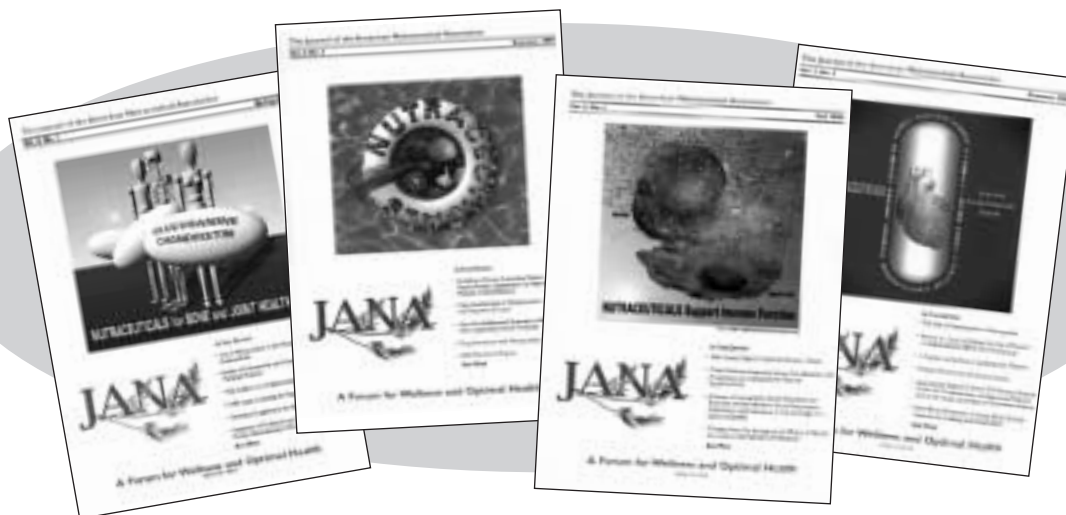
The use of β1,3-glucan is of special interest in the cancer patient undergoing chemotherapy and/or radiation treatment, since β-glucans have shown a remarkable ability to accelerate hematopoetic recovery in both sublethally and lethally irradiated mice, even when given after the radiation dose. It can also stimulate recovery of the bone marrow following chemotherapy, something vital to restricting tumor growth and preventing infectious complications during treatment.

While data provided in the research by Vetvicka and co-workers is preliminary and needs to be confirmed by a larger controlled trial, this is an important pilot study, in that it demonstrates the effectiveness of oral β1,3-glucan in treating both infectious agents and tumors.

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Pilot Study: Orally-Administered Yeast β1,3-glucan Prophylactically Protects Against Anthrax Infection and Cancer in Mice

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ABSTRACT

β1,3-glucans from various bacterial, mushroom, yeast, and cereal sources have been established as immunomodulators. In the present paper we demonstrate that orally-administered yeast β1,3-glucan (WGP Beta Glucan) had significant effects as a prophylactic treatment to reduce the mortality of anthrax infection in mice. In addition, the same type of treatment also inhibited the growth of cancer cells *in vivo*. The mechanism of action involves the stimulation of three important cytokines: IL-2, IFN-γ, and TNF-α. These results provide preclinical evidence for the beneficial effects of orally-administered yeast β1,3-glucan.

INTRODUCTION

β1,3-glucan's role as a biologically active immunomodulator has been well documented for over 40 years. First interest in the immunomodulatory properties of polysaccharides was raised after experiments showing that a crude yeast cell preparation stimulated macrophages via activation of the complement system.[1] Further work identified the immunomodulatory active component as β1,3-glucan.[2] Numerous studies have subsequently shown that β1,3-glucans, either particulate or soluble, exhibit immunostimulating properties, including antibacterial and anti-tumor activities.[3,4]

β1,3-glucans can be isolated from almost every species of yeast. β1,3-glucan derived from *Saccharomyces cerevisiae* (Baker's yeast) has been the most extensively studied. β1,3-glucan forms a significant part of the yeast cell wall, together with mannan, proteins, lipids, and small amounts of chitin. In addition to yeast, β1,3-glucans can be isolated from bacteria, mushrooms, algae, or cereal grains. The structure of the β1,3-glucan depends on both source and type of isolation. Different physicochemical parameters, such as solubility, primary structure, molecular weight, and branching play a role in the biological activities of β1,3-glucans.[5]

Original studies on the effects of β1,3-glucan on the immune system focused on mice. Subsequent studies demonstrated that β1,3-glucan has strong immunostimulating activity in a wide variety of other species, including earthworms, shrimps, fish, chicken, rats, rabbits, guinea pigs, sheep, pigs, cattle, and humans (for review see reference 6). Based on these results it has been concluded that β1,3-glucan represents a type of immunostimulant that is active across the evolutionary spectrum, likely representing an evolutionarily-conserved innate immune response directed against fungal pathogens.[7-9]

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More than 800 publications have reported that β1,3-glucans, either soluble or particulate, exhibit immunomodulatory properties. Despite the extensive investigations, no consensus on the source, size, and other properties of β1,3-glucan has been achieved. In addition, numerous concentrations and routes of administration have been tested, including intraperitoneal, subcutaneous, and intravenous applications. For decades, oral treatment with β1,3-glucan has been on the periphery of interest, despite the fact that it represents the most convenient route. In the last decade, however, a renewed interest in human application brought about important studies of orally-administered β1,3-glucan.

In this paper we report on the activity of orally-delivered yeast β1,3-glucan (WGP Beta Glucan) in infectious disease and tumor animal model systems. Although the anti-infective properties of β1,3-glucans have been already established, the majority of this work has been done with parenterally-administered β1,3-glucans, and the number of bacteria tested were very limited. Due to the recent threats of bioterrorism, we tested the effects of orally-delivered yeast β1,3-glucan on infection with *Bacillus anthracis.* Similarly, the anti-tumor effects of β1,3-glucans are well established, and most of the work has been done with parenterally-administered fungal β1,3-glucans, or oral administration of crude mushroom glucan preparations. In this study we tested the effects of oral administration of highly purified yeast β1,3-glucans on tumor growth.

MATERIAL AND METHODS

Yeast β1,3-glucan

A highly purified particulate yeast-derived β1,6-branched β1,3-glucan (Imucell™ WGP Beta Glucan, Biopolymer Engineering, Inc., Eagan, Minn.) was used in all experiments. The glucan was suspended in water at indicated concentrations.

Bacteria

Bacillus anthracis strain Vollum 1B (USAMRIID, Ft. Detrick, Md) was used for preparation of spores by harvesting from blood agar plates followed by heat shock (80° C for 11 minutes). Aliquots diluted in phosphate buffered saline (PBS) were stored at -80°C. A well-established model of anthrax infection in mice was used.[10]

Mice

Female, 6-wk-old BALB/c mice were purchased from Charles River Laboratories, St. Constant, Quebec, or male 6-wk-old BALB/c mice were purchased from Clea Japan, Inc. For anthrax experiments, mice were maintained inside the biosafety level 3 laboratory at DRES. Care and handling of mice followed guidelines set out by the Canadian Council on Animal Care.

Tumor cell line

Mouse intestinal tumor cell line Colon26 was passaged

in vivo in BALB/c mice.[11] Twenty-one days after inoculation, the tumors were excised, gently teased over stainless steel screens, washed in RPMI 1640 medium and resuspended in PBS (1×10^6 viable cells/ml).

Anthrax-protective prophylactic effects

Experimental groups (10 mice/group) were gavaged daily (days -7 to 0) with 0.1 ml of water containing either 0, 2 mg/kg or 20 mg/kg of WGP Beta Glucan. On day 0, 60 minutes after the final oral treatment, mice were infected subcutaneously with an LD_{60} dose of 85 ± 11 anthrax spores/animal. Confirmation of the doses was determined by seeding 0.1 ml of the same suspension on blood agar plates. All experimental animals were monitored twice daily post-infection for 10 days. At day 10, mortality had plateaued and the experiment was ended.

Tumor-protective effects

Tumor cells (1×10^5 viable cells in 0.1 ml) were injected into the abdominal wall of all animals on day 0. Groups of mice (8 mice/group) were gavaged daily with water or WGP Beta Glucan (28.4 mg/kg) for 21 consecutive days (days 1-21). Twenty-one days after tumor administration animals were sacrificed, tumors excised, and tumor weight measured.

Chemicals

Fetal calf serum and RPMI 1640 medium were purchased from Gibco BRL (Rockville, MD), HEPES, Concanavalin A, and lipopolysaccharide were purchased from Sigma (St. Louis, MO).

Cytokines

For evaluation of cytokine production, we incubated purified spleen cells from each animal (2×10^6 cells/ml in RPMI 1640 medium with 5% FCS) in wells of a 24-well tissue culture plate. After addition of 1 μg of Concanavalin A or 10 μg of LPS per well, cells were incubated for 72 hr in a humidified incubator (37°C, 5% CO_2). At the endpoint of incubation, supernatants were collected, filtered through 45 μm filter and tested for the presence of IL-2, IFN-γ and TNF-α. Levels of cytokines were measured by commercial ELISA in accordance with the protocol for Cytoscreen™ of BioSource International, Inc. (Camarillo, CA).

RESULTS

Orally-administered WGP Beta Glucan treatments showed significant anthrax-protective anti-infective effects. Under our experimental conditions, 5 out of 10 control mice survived the anthrax infection. Compared to this 50% control survival rate, prophylactic daily oral doses of WGP Beta Glucan (2 or 20 mg/kg) increased survival to 100% (Figure 1).

Orally-administered WGP Beta Glucan treatments also showed tumor-protective effects on tumor size and vascularization (Figure 2). Table 1 shows the effects of oral administration of WGP Beta Glucan on tumor weight. These

results show the beneficial effects of WGP Beta Glucan, as evidenced by a statistically significant decrease (-21%) in tumor weight at day 21 from 0.66 +/- 0.06 g in control mice to 0.52 +/- 0.06 g in β1,3-glucan-treated mice (P<0.05). In addition, the control animals were observed to be inactive and crouching, and had reduced body temperature in comparison to the WGP Beta Glucan-treated animals.

Evidence of orally-administered WGP Beta Glucan immunomodulatory activity was also demonstrated through effects on the production of three different cytokines, IL-2, IFN-γ, and TNF-α (Table 2). The production of these three cytokines was measured after a 72 hr *in vitro* incubation of spleen cells isolated from control and WGP Beta Glucan-administered animals. For all three tested cytokines, oral administration of WGP Beta Glucan resulted in significantly-increased cytokine levels (IL-2 (2.3-fold), IFN-γ (4.4-fold), and TNF-α (2.2-fold), P< 0.05) over control animals.

## DISCUSSION

β1,3-glucan is widely used as a dietary supplement, with well-established stimulating effects on the immune defense system.[6,12] A large body of published data supports this use. Browder *et al*. described strongly decreased septic morbidity with β1,3-glucan administration.[13] A series of well-documented multicenter blind studies demonstrated that β1,3-glucan-treated patients had significantly lower infection rates.[14,15] Positive effects were also found in patients after cardiopulmonary bypass,[16] and inhibition of

antiviral activity has been found in HIV-infected patients.[17] Some β1,3-glucans are routinely used in patients for tumor immunotherapy.[18,19]
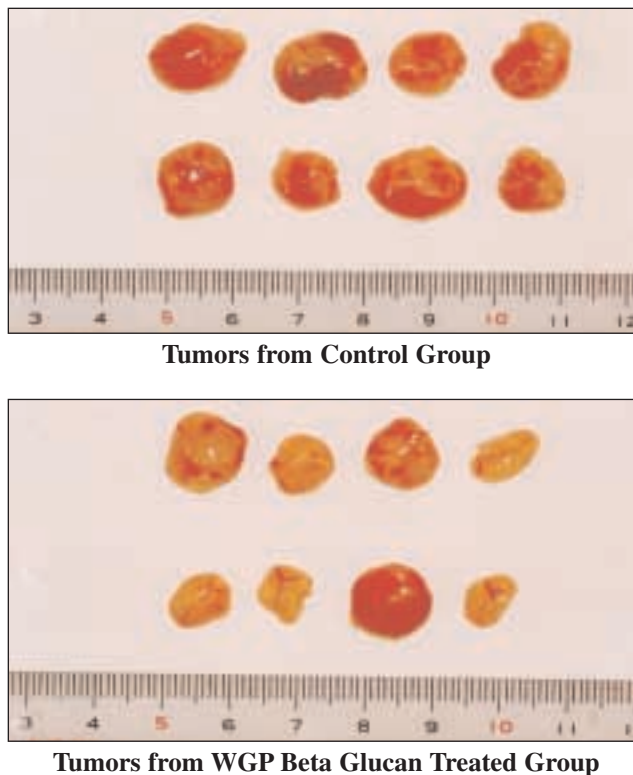
The majority of the previous work has dealt with injected β1,3-glucans. Only a limited number of investigations have focused on oral administration. Suzuki *et al*. demonstrated significant activation of peritoneal macrophages by orally-delivered β1,3-glucan.[20,21] In a subsequent communication they reported an enhancement of alveolar macrophage function by oral delivery of β1,3-glucan,[22] probably mediated via the Peyer's patches in the intestinal wall. Oral administration of lentinan has been found to increase the number of T helper cells in blood of lentinan-fed rats.[23] To further study the mechanism of action of orally-administered β1,3-glucans, Ikuzawa *et al*. studied the fate and tissue distribution of Krestin.[24] Based on these promising reports, latter attention focused on effects of β-glucans delivered *per os*. The summation of this work suggests that β1,3-glucans function by stimulating host immune defense mechanisms, primarily macrophages, neutrophils, and NK cells.

Figure 2. Tumor-protective effect of daily oral administration of WGP Beta Glucan**.**

Tumors from Control Group

Tumors from WGP Beta Glucan Treated Group

Figure 1. Anthrax-protective effect of daily oral prophylactic administration of WGP Beta Glucan.

Days Post-Infection
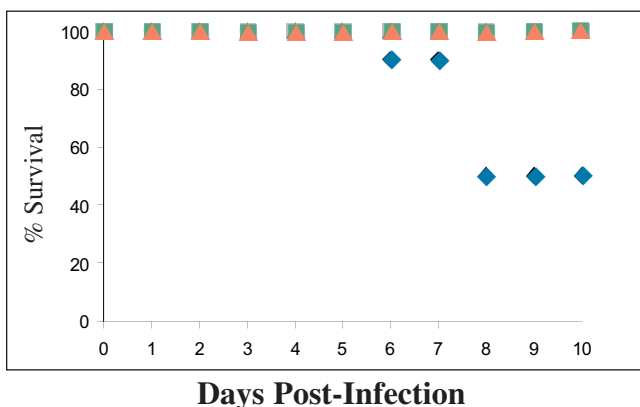
Groups of 10 Balb/c mice were gavaged daily (days –7 to 0) with 0.1 ml of water as a control (◆) or 0.1 ml water containing 40 (■) or 400 μg (▲) of WGP Beta Glucan per mouse (2 or 20 mg/kg). On Day 0, one hour after the last prophylactic oral dosing, animals were infected subcutaneously with an LD_{60} dose of *B. anthracis* spores. Animals were observed daily until the end of the study and survival time recorded. The percentage survival was calculated from the ratio of surviving animals each day to the total number of challenged animals in each group (n = 10). *P values were determined using a Fisher exact test (daily prophylactic 2 and 20 mg/kg; P = 0.016).

Tumor cells were implanted into all animals by abdominal wall injection on Day 0. Groups of mice (8 mice/group) were gavaged daily with water or WGP Beta Glucan (28.4 mg/kg) for 21 consecutive days (days 1 to 21). Twenty-one days after tumor administration animals were sacrificed, tumors excised and photographed.

Table 1. Effect of oral administration of WGP Beta Glucan on tumor growth

	Control	WGP Beta Glucan
Tumor Weight (g)	0.66 +/- 0.06	0.52 +/- 0.06*

At day 21 after tumor cell administration animals were sacrificed, tumors excised, and weighed. *P values were determined using a student's T-test (p<0.05).

With the threat of bioterrorism in the United States becoming a reality, we tested the hypothesis that oral yeast β1,3-glucan could be used as a protective agent against anthrax infection. The preclinical results described in this report demonstrate that orally-administered WGP Beta Glucan has strong anthrax-protective effects. Oral WGP Beta Glucan treatment significantly increased the number of surviving animals as well as prolonged survival time of lethally-infected animals. Dose ranging studies to date have demonstrated that daily prophylactic doses of 2-20 mg/kg WGP Beta Glucan provides a maximal anthrax-protective effect in mice. Ongoing dose ranging studies are being conducted to determine the minimal WGP Beta Glucan dose required to protect mice against a lethal anthrax infection. Based on the presented preclinical data, WGP Beta Glucan shows promise as a prophylactic treatment to support the immune system and reduce the risk of anthrax infection.

Oral WGP Beta Glucan treatment also reduces the threat of cancer, slowing down the progression of metastatic tumor growth in a preclinical colon cancer model. This observation extends the large body of preclinical and clinical work done in Japan demonstrating the oral anti-tumor activity of mushroom β1,3-glucans[25-27] to yeast β1,3-glucan. These published studies have demonstrated that β1,3-glucan immunotherapy leads to the activation of the innate immune cells (macrophages, neutrophils (PMN) and natural killer (NK) cells), the stimulation of tumoricidal activities, production of cytokines, and the generation of enhanced cell-mediated responses. Suzuki and colleagues have reported the stimulation of activated macrophages by the administration of SSG[22] and NK-type lymphokine-activated killer cells by the combined administration of lentinan and IL-2.[28] The stimulation of tumoricidal activities in PMN by a linear bacterial β1,3-glucan has also been reported.[29,30] A number of clinical studies have demonstrated synergy between oral β1,3-glucan immunotherapy, and traditional radiation and chemotherapeutic cancer treatment options.[31, 32]

At present we do not fully understand the mechanisms mediating the anthrax and tumor-protective effects of WGP Beta Glucan. We believe that through specific interactions between the β1,3-glucan active component of WGP Beta Glucan and β1,3-glucan receptors on M-cells within Peyer's patches in the intestinal mucosa that a systemic signal provided by cytokines is elicited by the gut-associated

Table 2. Effects of oral administration WGP Beta Glucan on cytokines

Cytokine	Control	WGP Beta Glucan
IL-2	9.7+/- 0.5 pg/ml	23.4 +/- 2.1 pg/ml*
IFN-γ	107.8 +/- 8.4 pg/ml	475.8 +/- 42.3 pg/ml*
TNF-α	487.8 +/- 58.2 pg/ml	1083.5 +/- 44.6 pg/ml*

Twenty-one days after tumor cell administration, animals were sacrificed, spleens excised, and spleen cells purified from each animal. Spleen cells from each animal were cultured with 1 μg of Concanavalin A or 10 μg of LPS for 72hr. Culture supernatants were collected, filtered through 45 μm filter, and tested for presence of IL-2, IFN-γ, and TNF-α. *P values were determined using a student's T-test (P<0.05 for IL-2, IFN-γ, and TNF-α).

lymphatic system that stimulates the innate immune system components (macrophages, neutrophils, and NK cells) to a higher functional level, increasing the first line of host defense mechanisms. For these experiments we focused on three important cytokines, IL-2, IFN-γ, and TNF-α. All of these cytokines play an important role not only in physiological processes, but also in bioregulation of host defense reactions. IL-2 is a cytokine produced by activated CD4 and some CD8 T lymphocytes. In addition to being the major T cell growth factor, IL-2 also stimulates: growth and differentiation of cytotoxic T cell precursors, NK cells, differentiation of activated human B-lymphocytes, and activation of monocytes. TNF-α is a pleiotropic cytokine secreted primarily by monocytes/macrophages and T lymphocytes, respectively. TNF-α is the principal mediator of natural immunity against gram-negative bacteria and a key mediator of inflammatory responses and septic shock.[33] IFN-γ, sometimes called immune interferon, is produced mainly by T lymphocytes as a result of antigenic or mitogenic stimulation. The activities of IFN-γ are many, including induction of MHC expression, macrophage activation, and effects on the differentiation of lymphocytes.

In this paper we report important biological activities of yeast-derived β1,3-glucan (Imucell™ WGP Beta Glucan). Oral administration of WGP Beta Glucan increased the production of three important cytokines (IL-2, IFN-γ, and TNF-α), inhibited growth of cancer cells *in vivo,* and provided a prophylactic defense against anthrax infection in mouse models. Despite our current lack of knowledge about the precise mechanisms through which oral β1,3-glucan mediates its protective effects, these anti-tumor and anti-infective properties of yeast-derived WGP Beta Glucan presented in this report suggest that further study is warranted to understand these benefits of β1,3-glucans.

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