

Original Article

Anthrax-Protective Effects of Yeast Beta 1,3 Glucans

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Abstract and Introduction

Abstract

Context: The recent events increasing the threat of bioterrorism have prompted a widespread search for defenses against this peril.

Objective: To evaluate the anthrax-protective effect of beta1,3-glucan immune modulators (PGG-glucan and WGP beta glucan) in an experimental animal model.

Design: Beta1,3-glucan immune modulators were administered by subcutaneous injection to Balb/c mice 2 days prior to anthrax challenge. WGP beta glucan was administered by daily oral gavage for 7 days prior to challenge, or in drinking water for 10 days postchallenge with a lethal dose of *Bacillus anthracis* spores. Survival, survival time, and microbial bioburden relative to an infected, untreated control group were assessed.

Results: A single injected dose of PGG-glucan or WGP beta glucan immune modulators given 2 days before challenge significantly: (a) increased the survival rate of infected mice (2.5-fold), (b) diminished the bacterial load in the lungs of infected mice (4-8-fold), and (c) increased the proportion of bacteria-free animals 10 days after challenge (2-fold). In mice prophylactically administered oral WGP beta glucan for 1 week prior to infection, survival increased from 50% to 100%; therapeutic administration of oral WGP beta glucan for 10 days postinfection increased survival from 30% up to 90% in treatment groups.

Conclusions: These results demonstrate the potential for beta1,3-glucan immune modulators to provide a significant degree of protection against anthrax, a potential biological warfare (BW) agent in a mouse model of anthrax infection. Further studies are needed to optimize protection, evaluate activity in combination with other treatment options, demonstrate activity in a validated primate model of infection, and determine if protection is effective against other potential BW agents.

Introduction

A recent national survey found that more Americans -- nearly two thirds -- are concerned about the possibility of a bioterrorism attack than they were in the weeks following September 11, 2001.^[1] One potential bioterrorism agent is *B anthracis*, which has been weaponized by several nations and killed 5 Americans in 2001. *B anthracis* is a very large aerobic Gram-positive rod-shaped micro-organism that commonly infects herbivorous animals, causing a serious and often fatal disease.

Anthrax spores are produced at temperatures below 30°C in soil and on inanimate objects. In humans, anthrax is very rare. The most common form of the disease in humans is cutaneous anthrax, which is usually acquired via injured skin or mucous membrane when in contact with spores or infected tissues. The spores germinate, vegetative cells multiply, and a characteristic gelatinous edema develops at the site. Death following treatment is very rare, but the untreated mortality rate is near 20%. Ingestion of undercooked meat may result in gastrointestinal anthrax. Nausea, vomiting, and gastrointestinal bleeding ensue within 12 to 18 hours, and infection spreads to the bloodstream and can be fatal. Inhalation of anthrax spores causes an influenza-like illness within 2 to 43 days of exposure.^[2] The inhalation form of anthrax is nearly always fatal because of the rapid progression of the disease and the gradual onset of flu-like symptoms.^[3]

The recent dissemination of anthrax spores through the mail has raised our awareness that we have relatively few prevention and treatment options to protect the US public. Conventional antimicrobial therapies, such as antibiotics, are useful to treat some bioterrorist threats, but their widespread prophylactic use is not recommended to protect the public due to the fear of antibiotic resistance.^[4] Prophylactic administration of vaccines provides an opportunity to protect the public from infection; however, a single vaccine does not provide broad protection against the many possible pathogenic bioterrorist threats. In the case of anthrax, the licensed human vaccine that consists of a series of 6 doses with yearly boosters is limited to military use and those at high risk of exposure (eg, laboratory workers, veterinarians).^[5] Further, there remain the issues of the timeframe for the development of safe and effective vaccines, or treatments, and the cost-effective delivery of these treatments to a large military or civilian population.

The use of immune modulators, either as a prophylaxis and/or as part of a treatment regimen following exposure, may represent a broad-spectrum approach to protect the public when exposed to a pathogenic challenge.^[6] Immune modulators can increase the nonspecific components of the immune system, such as the macrophage/neutrophil innate immune response, and stimulate the maturation of myeloid progenitor cells. Macrophage/neutrophil activation is largely considered the first line of defense against bacterial, fungal, and some viral infections. These cells are also necessary for the efficient presentation of antigens, and their level of activation determines the efficacy of the acquired immune system, the second line of defense leading to effective humoral (antibody) and cellular (T-cell) immune responses.^[7] Thus, immune modulators have the potential to enhance the effectiveness of other medical countermeasures, such as vaccines, monoclonal antibodies, and antibiotics.

One class of extensively studied immune modulators is known as the beta1,3-glucans. Beta1,3-glucans are carbohydrate polymers purified from yeast, mushroom, bacteria, algae, or cereals.^[8] The chemical structure of beta1,3-glucan is dependent on the source; different physicochemical parameters, such as solubility, primary structure, molecular weight, and branching, play a role in biological activities of beta1,3-glucans.^[9] Beta1,3-glucan works, in part, by stimulating the innate antifungal immune mechanisms to fight against a range of pathogenic challenges, including bacterial, fungal, parasitic, and viral infections.

Yeast beta-glucans are produced in 2 forms, an insoluble particle (whole glucan particle (WGP) beta glucan) and a solution (PGG-glucan). WGP beta glucan is purified from

baker's yeast cell walls following extraction of cellular proteins, nucleic acids, lipids, and most nonglucose-based oligosaccharides (eg, chitins, mannans) by a morphologically nondestructive proprietary process.^[10] What remains is a highly purified, 3-5 micron, spherical beta1,3-glucan particle. PGG-glucan (poly-1-6-beta-D-glucopyranosyl-1-3-beta-D-glucopyranose) is a highly purified soluble glucose polymer prepared by acid hydrolysis of WGP beta glucan.^[11] Both forms of beta1,3-glucan have been studied in preclinical and clinical studies.^[12] The mechanism of action of systemically administered beta1,3-glucan has been established in a series of published studies. Beta1,3-glucan binds to beta-glucan receptors present in the membranes of phagocytic cells and initiates a cascade of events leading to the expression of an overall heightened cellular immune response.^[13,14] This response also includes the proliferation of white blood cells in the bone marrow, leading to higher levels of these cells in the body, and an increased immune functionality of these cells.^[15] It is believed that this enhancement of macrophage and neutrophil function by beta1,3-glucan leads to the observed enhancement in microbial clearance and reduction in mortality of lethally infected animals.^[16,17] The oral protective effect of beta1,3-glucan appears to be mediated through receptor-mediated interactions with M cells found in Peyer's patches in the intestinal mucosa leading to the stimulation of cytokine production (interleukin-2, interferon-beta, tumor necrosis factor-alpha), enhanced resistance to infection, and an enhanced antitumor immune response.^[18-21]

This report evaluates the potential of the immune modulators PGG-glucan and WGP beta glucan to enhance resistance against a lethal systemic *B anthracis* spore infection in a mouse model system.

Materials and Methods

Animals

Six-week old, female Balb/c mice weighing 14-16 g (Charles River Laboratories, St. Constant, Quebec, Canada) were used in this study. Animals were maintained at a maximum of 5 mice per cage under standard laboratory conditions, and water and food were given *ad libitum*. Handling of animals was performed inside BL-3 Fume hoods, on secured animals. All protocols used in these experiments were approved by the Defence R&D Canada (DRDC) Institutional Animal Care Committee under protocol BK 01-01 and were cared for according to the Canadian Council on Animal Care, Guide to the Care and Use of Experimental Animals, vol. 1, second edition.

Bacteria

B anthracis Vollum 1B, a virulent, encapsulated, toxin-producing strain (obtained from USAMRIID, Ft. Detrick, Maryland) was used in these studies in the BL-3 facilities at DRDC. *B anthracis* spore stocks were prepared by harvesting a cell suspension from a blood agar plate (BAP) in phosphate buffered saline (PBS). The cell suspension was heat shocked at 80° C for 11 minutes in PBS to kill vegetative cells. Aliquots were stored at -80° C. The frozen spore stock was diluted and used in the protection studies.

Immune Modulators

WGP beta glucan (*Imucell*/WGP beta glucan, Biopolymer Engineering Inc., Eagan, Minnesota) was purified from the cell walls of baker's yeast. PGG-glucan (Alpha-Beta Technology Inc., now available from Biopolymer Engineering, Inc. Eagan, Minnesota) is a highly purified soluble glucose polymer prepared by acid hydrolysis from WGP beta glucan.

Protection Studies

To test the prophylactic effects of systemic PGG-glucan or WGP beta glucan treatment, groups of mice ($n = 10$) were administered single subcutaneous (SQ) doses of PGG-glucan (50 mcg/mouse in 0.1 mL of saline), WGP beta glucan (200 mcg/mouse in 0.1 mL of saline) or a saline control in the flank 2 days prior to challenge with an LD₇₀B *anthracis* spore challenge, SQ. Confirmation of the infectious doses was achieved by seeding 0.1 mL of the suspension used for infection on BAP. Each systemic administration experiment was carried out twice.

The oral prophylactic anthrax-protective effects of WGP beta glucan were tested by administering a WGP beta glucan suspension (40 or 400 mcg/mouse, or 2 or 20 mg/kg) in water by gavage (daily days -7 to 0, or 4 times a week days -7, -4.5, -2, and 0). To comply with worker safety requirements prohibiting the handling of anthrax-infected animals, the therapeutic oral protective effects of WGP beta glucan were tested by administering WGP beta glucan as a 0.3% w/v carboxymethylcellulose (CMC-P325G, PL Thomas, Morristown, New Jersey) suspension in the drinking water (daily days 0 to +10) at WGP beta glucan concentrations calculated to deliver daily doses of 0, 40, or 400 mcg per mouse/day based on the estimated drinking water consumption of 6.5 mL water/mouse/day. Actual dosing was determined by daily measurement of water consumption, factoring the number of live animals per cage each day, and was calculated to be 0, 22.6 ± 3.5 and 200.3 ± 36.4 mcg per mouse/day, or 0, 1.5, or 13.3 mg/kg. Control groups received either vehicle gavage or carboxymethylcellulose in their drinking water only. On day 0, one hour after the oral dosing, animals were infected SQ with an LD₆₀ dose of anthrax spores. Animals were observed daily until the end of the study (day 10) and survival time was recorded. Percent survival was calculated from the ratio of surviving animals each day to the total number of infected animals in each group ($n = 10$). Each oral dosing experiment was carried out once.

Microbial Clearance

At the time of death, or 10-11 days following challenge, surviving animals were sacrificed, and the lungs harvested, weighed, and homogenized in 20 mL of PBS for bacterial counts. Lung homogenates were serially diluted and 0.1 mL seeded onto BAP and incubated for 24 hours at 35°C in order to evaluate the number of colony-forming units (CFU) per gram lung tissue. Each experiment was repeated once and the *P* values were determined using a student's T-test.

Results

The anti-infective effects of the beta1,3-glucan immune modulators PGG-glucan and WGP beta glucan were tested in a well-established mouse model of systemic anthrax infection.^[22] As shown in Figure 1, systemic prophylactic treatment with a single dose of PGG-glucan (SQ) significantly increased the number of survivors from 7 of the 22 control

mice (31.8%) to 19 of the 22 PGG-glucan-treated mice (86.4%, $P = .00005$). WGP beta glucan (SQ) also significantly increased the number of survivors from 31.8% in the control groups to 18 of the 22 WGP beta glucan-treated groups (81.8%, $P = .00024$).

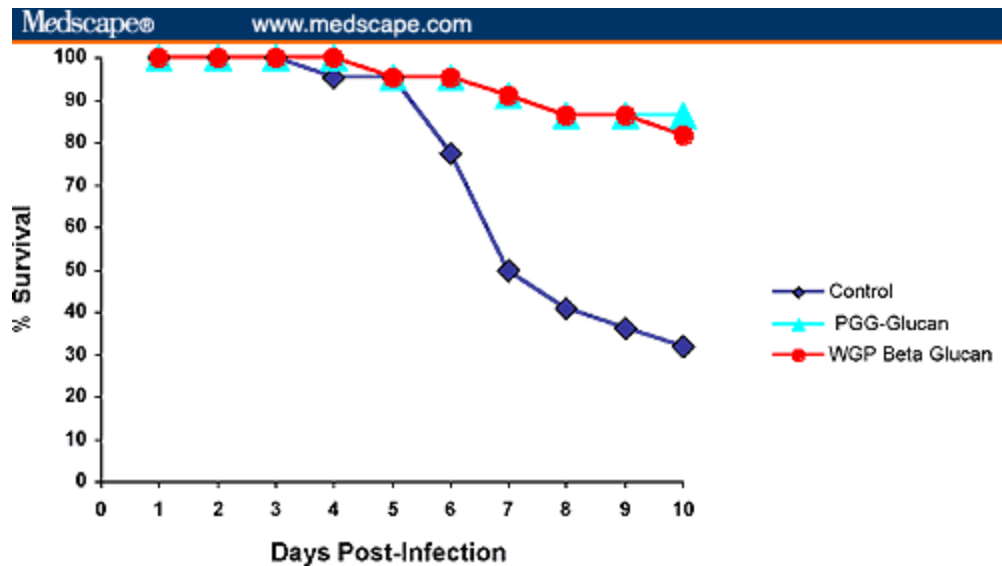
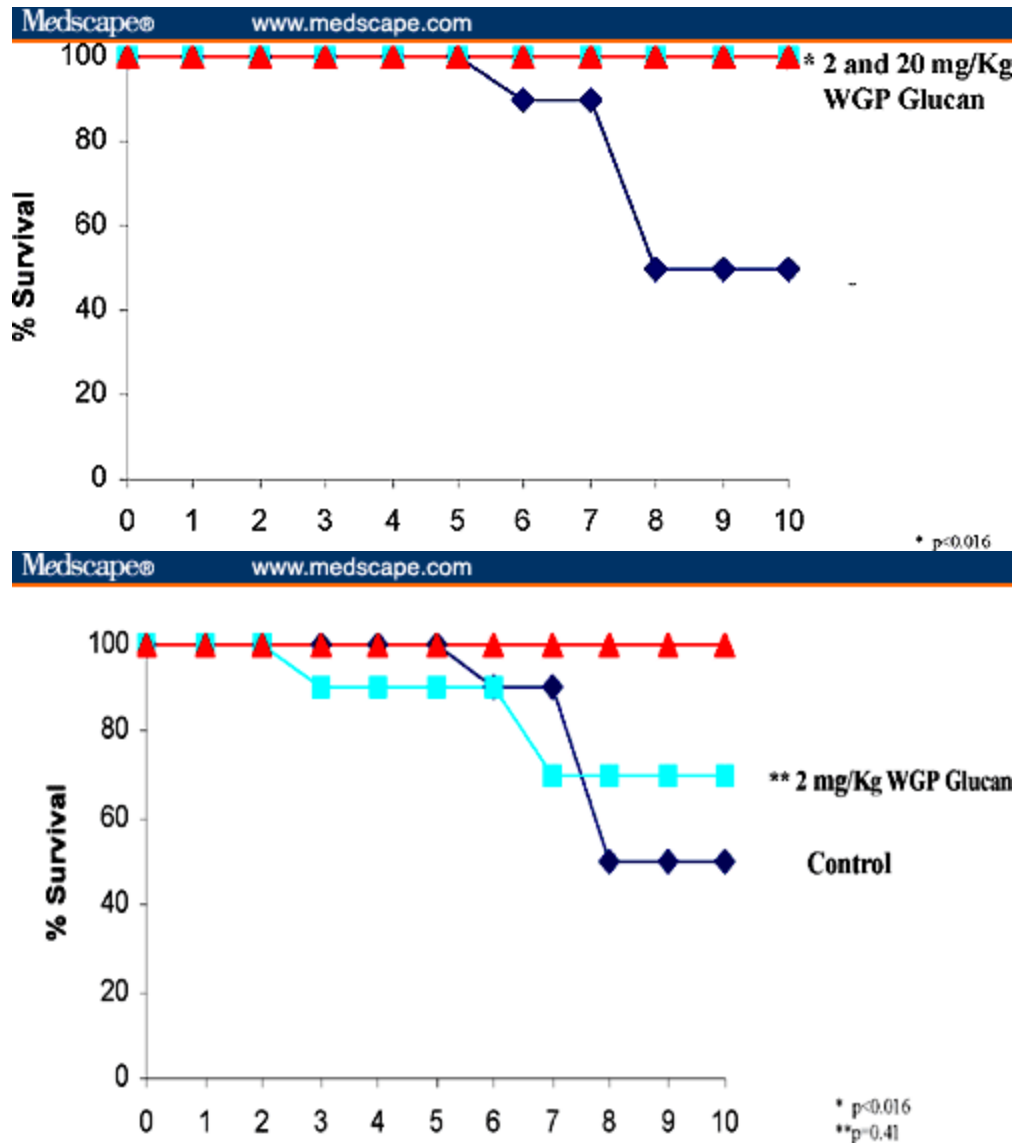


Figure 1. Anthrax-protective effects of systemic prophylactic treatment with beta1,3-glucans. A single dose of PGG-glucan (red circle 1 mg/kg) or WGP beta glucan (turquoise triangle 10 mg/kg) or control saline (blue diamond) was administered 2 days before a lethal challenge with *B anthracis*. The course of the infection was followed for a period of 10 days. During this period, the number of survivors was recorded daily. The percentage survival was calculated from the ratio of surviving animals each day to the total number of challenged animals in each group. Pooled data from duplicate experiments were used. * P values were determined using a Fischer exact test (PGG-glucan, $P = .00005$; WGP beta glucan, $P = .00024$).

The mechanism of action of these anthrax-protective immune modulators by the stimulation of the host innate antimicrobial immune response resulted in enhanced microbial killing, as evidenced by a significantly reduced microbial bioburden in the lungs of treated animals (Table 1). This enhancement of the host innate immune response also resulted in a significant percentage (>80%) of the surviving treated anthrax-challenged mice to be bacteria-free by 10 days post-challenge, as seen by an absence of *B. anthracis* CFU in the lung.

The survival results shown in Figure 2A demonstrate that daily oral prophylactic dosing of WGP beta glucan (> 2 mg/kg) also significantly increased the number of anthrax survivors. In these experiments, 5 of 10 control animals survived the anthrax infection (50% survival), but animals treated prophylactically with daily oral doses of 2 or 20 mg/kg WGP beta glucan showed 100% survival ($P = .016$). In contrast, 4-times-weekly oral prophylactic dosing at 2 mg/kg was not as effective as daily dosing ($P = .41$); a 20 mg/kg WGP beta glucan dose was required to achieve significant protection ($P = .016$) (Figure 2B).



Days Post-Infection

Figure 2. Effect of prophylactic oral WGP beta glucan treatment regimen on survival to a lethal anthrax challenge. Oral doses of WGP beta glucan (0 [*blue diamond*], 2 mg/kg [*turquoise square*], or 10 mg/kg [*red triangle*]/mouse) were gavaged (daily, days -7 to 0) (Figure 2A) or 4 times a week (days -7, -4.5, -2, 0) (Figure 2B) before lethally challenged with *B anthracis* on day 0. The course of the infection was followed for a period of 10 days. Percent survival was calculated from the ratio of surviving animals each day to the total number of infected animals in each group (n = 10) and compared with a water gavage control group. *P values were determined using a Fischer exact test (daily prophylactic 2 and 20 mg/kg, P = .016; 4 times weekly prophylactic 2 mg/kg, P = .41; 20 mg/kg, P = .016).

Daily oral therapeutic dosing of WGP beta glucan (> 1.5 mg/kg) also significantly increased the number of anthrax survivors (Figure 3). In these experiments, 3 of 10 control animals survived the anthrax infection (30% survival). In comparison, at the 1.5-

mg/kg WGP beta glucan oral therapeutic dose level, 80% of the treated mice survived ($P = .038$), and at the 13.3 mg/kg WGP beta glucan oral therapeutic dose level, 90% of the treated mice survived ($P = .01$).

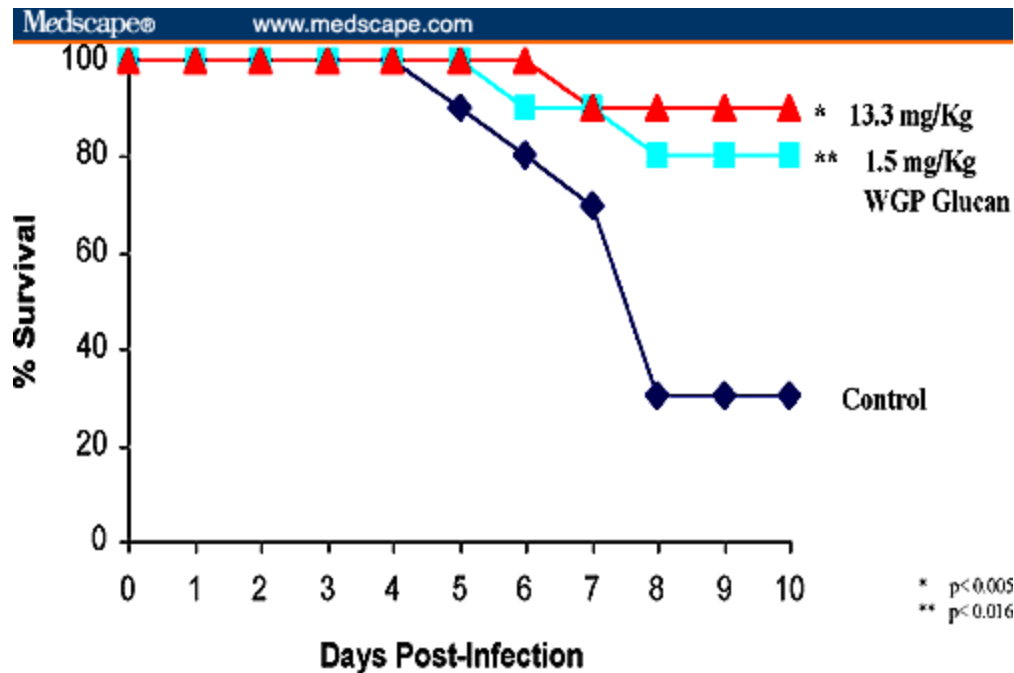


Figure 3. Effect of therapeutic oral WGP beta glucan treatment regimen on survival to a lethal anthrax challenge. Groups of 10 animals were lethally infected with *B anthracis* spores. One hour after infection, oral therapeutic doses of WGP beta glucan (0 [blue diamond], 1.5 mg/kg [turquoise square], or 13.3 mg/kg [red triangle]/mouse) were administered in the drinking water (days 0 to +10). The course of the infection was followed for a period of 10 days. Percent survival was calculated from the ratio of surviving animals each day to the total number of infected animals in each group, and compared with a water control group. * P values were determined using a Fischer exact test (1.5 mg/kg, $P = .038$; 13.3 mg/kg, $P = .01$).

Discussion

Seemingly unthinkable before September 11, the threat of bioterrorism in America has become a real and terrifying danger to the public. Under best conditions, the expected survival rate of symptomatic people unknowingly exposed to lethal airborne dosages of anthrax is only 20% to 30% using traditional therapies.^[23,24] Antibiotics, such as ciprofloxacin and doxycycline, are useful to treat anthrax.^[25] However, even with the use of multiple antibiotics, infected symptomatic patients have still succumbed to infection. Widespread prophylactic antibiotic use has been employed with high-risk populations but is not recommended to protect the general public.^[4] Prophylactic administration of the anthrax vaccine provides an opportunity to protect the public from anthrax infection. However, there is limited availability and debate as to whether the risk of adverse events justifies its general use.^[26,27]

Results presented in this paper suggest that immune modulation is an alternative strategy to broadly protect the military and the public from infection by anthrax. Both

systemically and orally administered beta1,3-glucans significantly increased the survival of anthrax-challenged animals. Beta1,3-glucan's role as a biologically active immune modulator has been well documented for more than 40 years, stemming from initial observations that a crude yeast cell preparation stimulated macrophages via activation of the complement system.^[28] Subsequent studies have shown that the immune modulatory active component beta1,3-glucan exhibits immunostimulating properties, including anti-infective and antitumor activities.^[29,30] Browder and colleagues^[31] described strongly decreased septic morbidity with beta1,3-glucan administration. A series of well-documented multicenter blind studies demonstrated that beta1,3-glucan-treated patients had significantly lower infection rates.^[32,33] Some beta1,3-glucans are routinely used in patients for tumor immunotherapy.^[34,35]

The anti-infective mechanism of action of beta1,3-glucan is mediated via the stimulation of the microbicidal activity of circulating blood cells (mainly monocytes/macrophages/dendritic cells, neutrophils, and natural killer cells) of the innate immune system. This involves interactions with a series of protein and glycolipid receptors present on the membranes of phagocytic white blood cells.^[36-39] Upon beta1,3-glucan binding, these receptors become crosslinked, initiating a selective cascade of cellular responses. This response also includes the proliferation of white blood cells in the bone marrow, leading to higher levels of these cells in the body, and an increased immune functionality of these cells.^[15] The documented increase in immune functionality includes increased ability of immune cells in circulation to recognize and migrate to a distal site of pathogenic challenge, increased phagocytic capacity,^[40] and increased production of endogenous antimicrobial agents, such as reactive oxygen intermediates,^[41] leading to an enhanced ability of the immune system to resolve a pathogenic challenge.

The oral protective effect of yeast beta1,3-glucan is believed to be mediated by a receptor-mediated uptake through M cells in the Peyer's patches in the intestinal wall, leading to WGP presentation to the macrophage and dendritic cells found in the underlying gut-associated lymphatic tissue. In 1990, Suzuki and colleagues^[19] demonstrated significant activation of peritoneal macrophages by orally delivered beta1,3-glucan.^[19] In a subsequent communication, they also reported enhancement of alveolar macrophage function by orally delivered beta1,3-glucan.^[42] Oral administration of lentinan, isolated from shiitake mushrooms, was found to increase the number of T-helper cells in blood of lentinan-fed rats.^[43] Oral beta1,3-glucan was shown to have anti-infective^[18,20] and antitumor activities in both preclinical and clinical studies.^[21,44,45] Based on all available data, beta1,3-glucans is believed to function by stimulating host immune defense mechanisms, primarily macrophages, natural killer cells, dendritic cells, and neutrophils, therefore enhancing microbial clearance and subsequently reducing mortality in lethally challenged animals.^[16,17]

Macrophage/neutrophil activation is largely considered the first line of defense against bacterial, fungal, and some viral infections, and plays a key role in *B anthracis* pathogenesis. These innate immune cells are the first to interact with *B anthracis* via phagocytosis and induce a host defense response, such as cell-mediated cytotoxicity and cytokine secretion. Guidi-Rontani and colleagues^[46] have demonstrated that vesicles derived from the phagosomal compartment of alveolar macrophages are the primary sites of spore germination. The early onset of toxin gene expression after germination is a key determinant in the macrophage response. The failure of the macrophage to kill the

germinating spore leads to bacilli replication and the production of the lethal factor protein, which is lytic for macrophages.^[47] It is proposed that the enhanced microbial killing capability of WGP beta glucan-stimulated macrophages and neutrophils results in the rapid ingestion and killing of anthrax spores before germination and toxin production, preventing bacterial replication, systemic disease, and death.

Macrophages and dendritic cells are also necessary for efficient antigen presentation, and their level of activation can determine the effectiveness of the humoral and cellular adaptive immune responses. The use of WGP beta glucan either in prophylaxis before exposure, and/or as part of a treatment regimen following exposure, represents 2 strategies that could provide clear health benefits whether the targeted population is the general public or the militaries exposed to a pathogenic challenge. Further, beta1,3-glucan has clearly been shown to enhance the effectiveness of other medical countermeasures, such as vaccines and antibiotics.^[48,49] The increase in survival time of mice prophylactically or therapeutically treated with WGP beta glucan suggests that immune modulator intervention could also provide time for other antimicrobial therapies to be started and become effective.

The results of these studies provide clear preclinical evidence that a daily dose of WGP beta glucan could provide a significant degree of protection against potential bioterroristic pathogenic agents, such as anthrax. Investigations of the protective effects of WGP beta glucan in a nonhuman primate model of anthrax infection, evaluations of the combined prophylactic and therapeutic protective effect, definitions of the time of onset and duration of the protective effect, and evaluations of synergy with antibiotic, antibody, and vaccine therapies are currently in progress.

Tables

Table 1. Enhancement of *B Anthracis* Microbial Clearance by Treatment With Systemic Prophylactic Beta1,3 Glucans

Treatment	CFU/g Lung	% Bacteria-Free Animals*
Control	5.4 X 10 ⁶	40.9
PGG-glucan	6.0 X 10 ⁵ (†.03)	86.4 (†.04)
WGP beta glucan	1.43 X 10 ⁵ (†.04)	90.9 (†.02)

Single doses of PGG-glucan (1 mg/kg), WGP beta glucan (10 mg/kg), or saline control were administered 2 days before a lethal challenge with *B anthracis*. The course of the infection was followed for a period of 10 days. At the end of the study, survivors were sacrificed and the bacterial loads in the lungs were evaluated. Results are reported as colony forming units (CFU) per gram of lung tissue.

*Limit of detection 100 CFU/g lung.

†P values were determined using a student's T-test.

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