BTR2003: Unified Science & Technology for Reducing Biological Threats and Countering Terrorism
March 2002

POTENTIAL FOR BETA 1,3-GLUCANS TO PREVENT AND TREAT BIOLOGICAL WARFARE INFECTIONS

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ABSTRACT

The new awareness of the threat of bioterrorism has prompted a widespread search for defenses against this menace. This paper introduces the concept of immune modulator use to reduce the peril of bioterrorism and infectious disease.

Results from the Mouse-Anthrax experiments detailed in this paper demonstrate that both prophylactic and therapeutic administration of the beta 1,3-glucan immune modulators: PGG-Glucan (PGG) and WGP Beta Glucan (WGP) significantly increased the survival rate of anthrax-infected mice. In animals prophylactically administered either systemic PGG or WGP survival increased from 30% in control-infected animals to greater than 80% in immunomodulator treated animals. In animals prophylactically administered oral WGP survival increased from 50% in control animals up to 100% in treated animals; therapeutically administered oral WGP survival increased from 30% in control-infected animals up to 90% in immunomodulator treated animals. The established mechanism of action for these immune modulators led to an enhancement of anthrax microbial clearance, as demonstrated by a significant decrease in bacterial load in the lungs of treated infected mice, and a significant increase in the proportion of bacteria-free mice 11 days after infection.

These results provide preclinical “proof-of-concept” that the beta 1,3-glucan immune modulators, PGG-Glucan and WGP Beta-Glucan show potential to provide a significant degree of protection against the morbidity and mortality of a potential bioterroristic attack.

INTRODUCTION

The recent increased threat of bioterrorism as evidenced by the dissemination of anthrax through the US postal system has raised our awareness that we have relatively few prevention and treatment options to protect the U.S. public. *Bacillus anthracis* has been weaponized by several nations in the past and is the most commonly mentioned potential bioterrorist agent [1]. *B. anthracis* is a large aerobic gram-positive rod-shaped microorganism that commonly infects herbivorous animals causing a serious and often fatal disease. Anthrax is endemic worldwide, but the incidence in North America and Europe is low with the availability of an effective vaccine [2].

Conventional anti-microbial therapies, such as antibiotics, are useful to treat some bioterroristic threats, but their widespread prophylactic use is not recommended because of the potential risk of selecting for antibiotic-resistant pathogens [3]. Prophylactic administration of vaccines provides an opportunity to protect the public from infection, however a single vaccine is only able to protect against infection by a single microorganism, and does not provide broad protection against the many possible pathogenic terrorist threats. In the case of anthrax, the licensed human vaccine that consists of a series of six doses with yearly boosters is limited to military use and those at high risk of exposure (e.g., laboratory workers, veterinarians) [4]. Further, there remain the issues of the timeframe for the development of these safe and effective vaccines, or treatments, and the cost-effective delivery of these treatments to a large military or civilian population.

An alternative approach to broadly protect the public from infection by a wide range of pathogenic microorganisms is through the enhancement of the immune system with immune modulators [5]. Immune modulators can increase specific 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 foreign antigens, and their level of activation can determine the efficacy of the acquired immune system, the second line of defense leading to effective humoral (antibody) and cellular (T-cell) immune responses [6]. The use of such modulators either as a prophylaxis before exposure and/or as part of a treatment regimen following exposure, represent two strategies that could provide benefits whenever the public is exposed to a pathogenic challenge. Further, 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 immunomodulators is known as the beta 1,3-glucans. Beta 1,3-glucans are carbohydrate polymers purified from yeast, mushroom, bacteria, algae, or cereal [7]. The chemical structure of beta 1,3-glucan is dependent on the source, and based on extensive published research it is widely accepted that the beta 1,3-glucan purified from baker's yeast (*Saccharomyces cerevisiae*) is the most potent anti-infective beta-glucan immunomodulator [8]. Yeast-derived beta 1,3-glucans work, in part, by stimulating the innate anti-fungal immune mechanisms to fight a range of pathogenic challenges, including bacterial, fungal, parasitic and viral infections.

Yeast beta-glucans are produced in two forms, an insoluble particle (Whole Glucan Particle, WGP Glucan) and a solution (PGG-Glucan). Both forms have been studied in preclinical and clinical studies [reviewed in 8]. The mechanism of action of systemically administered beta 1,3-glucan has been established in a series of published and unpublished studies. Beta 1,3-glucan binds to a beta-glucan receptors present on the membranes of phagocytic cells and initiates a cascade of events leading to the expression of an overall heightened cellular immune response [9-10]. This response also includes the proliferation of phagocytic 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 [11]. It is believed that this

enhancement of macrophage and neutrophil function by beta 1,3-glucan leads to the observed enhancement in microbial clearance and reduction in mortality of lethally infected animals [12-13].

This report details the studies to evaluate the potential of the immune modulators PGG-Glucan and WGP Glucan to enhance host resistance against a lethal *B. anthracis* infection in a mouse model system.

MATERIALS AND METHODS

Animals

Six-week old, female Balb/c mice weighing 14-16g (Charles River Laboratories, St. Constant, Quebec) were used in this study. Animals were maintained at a maximum of 5 mice/cage under standard laboratory conditions, and water and chow were given *ad libitum*. Handling of animals was performed inside Biosafety Fume hoods, on secured animals. All protocols used in these experiments were approved by the DRES Institutional Animal Care Committee (IACC) 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, 2nd edition.

Bacteria

Bacillus anthracis strain Vollum 1B (obtained from USAMRIID, Ft. Detrick, MD, USA) was used in these studies in the Bioconfinement 3 facility at DRES. *B. anthracis* spore stocks were prepared by harvesting a cell suspension in phosphate buffered saline (PBS) from a blood agar plate (BAP). The cell suspension was heat shocked at 80^{o}C for 11 min in PBS to kill vegetative cells and aliquots stored at -80^{o}C. The frozen spore stock was diluted and used in the protection studies.

Immune Modulators

WGP Glucan (Imucell™ WGP Glucan, Biopolymer Engineering Inc., Eagan, MN, USA) was purified from the cell walls of Baker's yeast following extraction of cellular proteins, nucleic acids, lipids, and most non-glucose-based oligosaccharides (e.g., chitin and mannans) by a morphologically non-destructive proprietary process [14]. What remains is a highly purified, 3-5 micron, spherical beta-glucan particle. PGG-Glucan (poly-1-6-β-D-glucopyranosyl-1-3- β-D-glucopyranose, Alpha-Beta Technology, now available from Biopolymer Engineering, Inc. Eagan, MN, USA) is a highly purified soluble glucose polymer prepared by acid hydrolysis from WGP Glucan that enhances specific humoral and cellular responses to challenge by pathogenic microorganisms [15].

Protection Studies

To test the prophylactic effects of systemic PGG-Glucan or WGP Glucan treatment groups of mice (n=10) were administered single subcutaneous (s.c.) doses of PGG-Glucan (50 μg/mouse in 0.1 ml of saline), WGP Glucan (200 μg/mouse in 0.1 ml of saline) or saline control in the flank two days prior to challenge with an LD_{70} *B. anthracis* spore challenge, s.c. 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 prophylactic oral anthrax-protective effects of WGP Glucan were tested by administering a WGP Glucan suspension (40 or 400 μg/mouse) in water by gavage (daily days –7 to 0, or four times a week

days -7, -4.5, -2, 0). The therapeutic oral protective effects of WGP Glucan were tested by administering WGP Glucan as a 0.3% w/v carboxymethylcellulose (CMC-P325G, PL Thomas) suspension in the drinking water (daily days 0 to + 10) at WGP Glucan concentrations calculated to deliver daily doses of 0, 40 or 400 μg 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 $\pm$ 3.5 and 200.3 $\pm$ 36.4 μg per mouse/day. Therapeutic administration in the drinking water was required because of the hazard of handling anthrax-infected animals. 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 s.c. with an LD_{60} dose anthrax spores. Animals were observed daily until the end of the study (day 10-11) and survival time 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. *p values were determined using a student's T-test.

Microbial Clearance

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

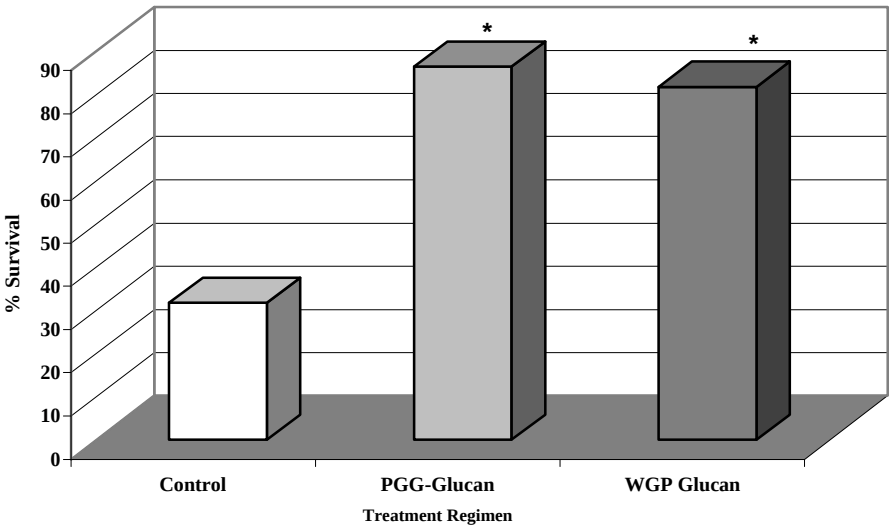
RESULTS

The anti-infective effects of the beta 1,3-glucan immune modulators, PGG-Glucan and WGP Glucan were tested in a well-established mouse systemic anthrax infection model system [16]. As shown in Figure 1, systemic prophylactic treatment with a single dose of PGG-Glucan 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 = 0.00005). WGP Glucan (s.c.) also significantly increased the number of survivors from 31.8% in the control groups to 18 of the 22 WGP Glucan-treated groups (81.8%, p= 0.00024).

The mechanism of action of these anthrax-protective immune modulators, 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 by 10-11 days post-challenge to be completely cured 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

Glucan (>2 mg/Kg) also significantly increased the number of anthrax survivors. In these experiments, 5 out of 10 control animals survived the anthrax infection (50% survival). In comparison, animals treated prophylactically with daily oral doses of 2 or 20 mg/Kg WGP Glucan showed 100% survival (p = 0.016). In contrast, four times weekly oral prophylactic dosing at 2 mg/Kg was not as effective as daily dosing (p = 0.41), requiring a 20 mg/Kg WGP Glucan dose to achieve significant protection (p = 0.016)(Figure 2B).
Daily oral therapeutic dosing of WGP Glucan (> 1.5 mg/Kg) also significantly increased the number of anthrax survivors (Figure 3). In these experiments 3 out of 10 control animals survived the anthrax infection (30% survival). In comparison, at the 1.5 mg/Kg WGP Glucan oral therapeutic dose level, 80% of the treated mice survived (p= 0.038), and at the 13.3 mg/Kg WGP Glucan oral therapeutic dose level, 90% of the treated mice survived (p = 0.01).

Figure 1: Anthrax-Protective Effects of Systemic Prophylactic Treatment With Beta 1,3-Glucans



A single dose of PGG-Glucan (50 μg) or WGP Glucan (200 μg) was administered two days before a lethal challenge with *B. anthracis*. The course of the infection was followed for a period of 11 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. **P* values were determined using a student's T- test (PGG-Glucan, $p = 0.00005$; WGP Glucan, $p = 0.00024$).

DISCUSSION

Seemingly unthinkable before September 11, the threat of bioterrorism in America has become a real and terrifying danger to the public and its leaders. Under best conditions, the expected survival rate of symptomatic people unknowingly exposed to lethal airborne dosages of anthrax is only 20-30% using traditional therapies [17-18]. Antibiotics, such as ciprofloxacin and doxycycline are useful to treat anthrax [19-20]. However, even with the use of multiple antibiotics, infected symptomatic patients have still succumbed to infection [21]. Widespread prophylactic antibiotic use has been employed with high-risk populations but is not recommended to protect the general public from being infected [3]. 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 [22-23].

The results presented in this paper demonstrate that immune modulation is an alternative strategy to broadly protect the military and the public from infection by anthrax. Both systemically and orally administered WGP Glucan significantly increased the survival of anthrax-challenged animals. The reported mechanism of action of WGP Glucan is through the stimulation of the microbicidal activity

of white blood cells (mainly monocytes, macrophages, neutrophils and NK cells) of the innate immune system. This involves interactions with a series of protein and

Table 1: Enhancement of *B. Anthracis* Microbial Clearance by Treatment with Systemic Prophylactic Beta-1,3-Glucans

Treatment	CFU/Lung	% Bacteria-Free Animals
Control	**1.08×10^6**	**40.9**
PGG-Glucan	**1.28×10^5 (* 0.03)**	**86.4 (* 0.04)**
WGP Glucan	**2.73×10^5 (* 0.04)**	**90.9 (*0.02)**

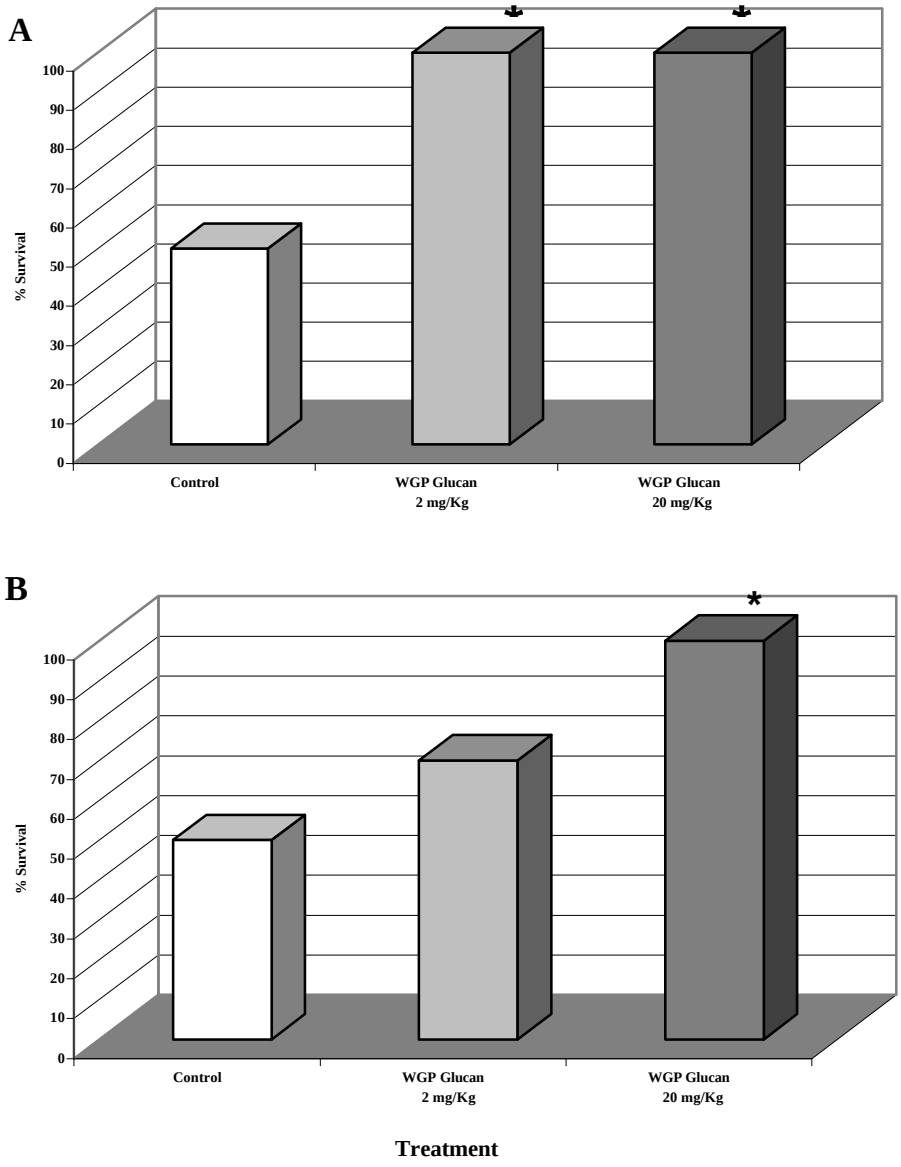
Single doses of PGG-Glucan (50 µg) or WGP Glucan (200 µg) were administered two days before a lethal challenge with *B. anthracis*. The course of the infection was followed for a period of 11 days. At the end of the study, survivors were sacrificed and the bacterial loads were evaluated from the lungs. **P* values were determined using a student's T-test.

glycolipid receptors present on the membranes of phagocytic white blood cells [24-28]. Upon WGP Glucan binding, these receptors become crosslinked, initiating a selective cascade of cellular responses. Early events, such as a Ca+ influx mediated by protein kinase C and activation of transcription factors, lead to the expression of an overall heightened cellular immune response [9-10]. This response also includes the proliferation of phagocytic 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 [11]. The documented increase in immune functionality includes increased cellular mobilization (ability of immune cells in circulation to recognize and migrate to a distal site of pathogenic challenge), increased phagocytic capacity (ability of the immune cells to engulf infectious bacteria, fungi, or a cancer cell)[29] and increased production of endogenous antimicrobial agents, such as reactive oxygen intermediates [30] leading to an enhanced ability of the immune system to resolve a pathogenic challenge. It is believed that this enhancement of macrophage and neutrophil functions by WGP Glucan treatment is responsible for the observed enhancement in microbial clearance and reduction in mortality in the lethally infected animals [12-13].

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 et al [31] have demonstrated that vesicles derived from the phagosomal compartment of alveolar macrophage are the primary sites of spore germination in a murine inhalation model. 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 (LF) protein, which is lytic for macrophages [32]. Hanna et al. [33] showed that sublytic concentration of LF could increase the production of IL-1 by activated macrophages. Such modification of inflammatory response may be stimulatory for the growth and diffusion of the bacterium. It is proposed that the enhanced microbial killing capability of WGP Glucan-stimulated

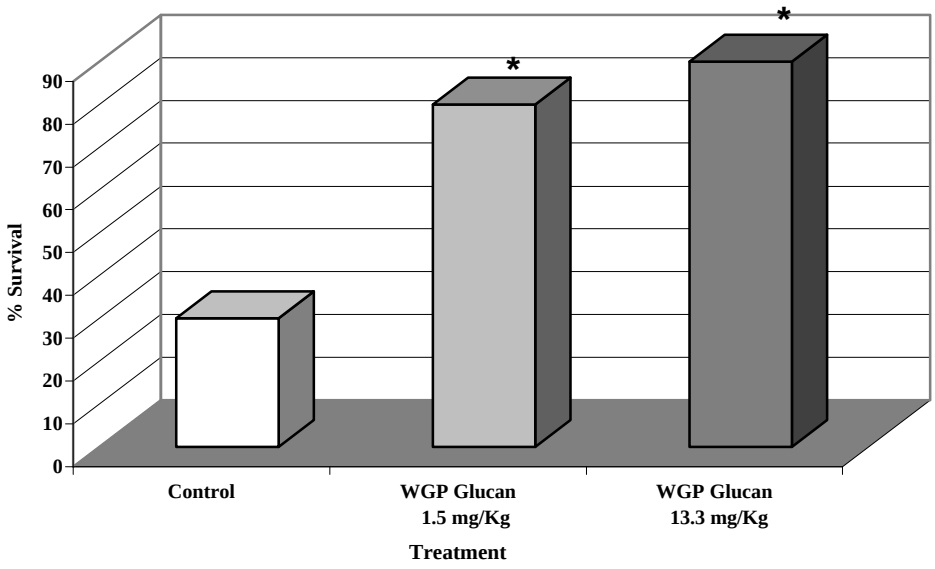
macrophages and neutrophils results in the rapid ingestion and killing of anthrax spores before germination and toxin production leading to bacterial replication, systemic disease and death.

Figure 2: Effect of Prophylactic Oral WGP Glucan Treatment Regimine on Survival to a Lethal Anthrax Challenge



Oral doses of WGP Glucan (0, 40 or 400 μg) were gavaged (daily, days –7 to 0) (Figure 2A) or four 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 to a water gavage control group. **p* values were determined using a student's T-test (Daily prophylactic 2 and 20 mg/Kg, p = 0.016; Four times weekly prophylactic 2 mg/Kg, $p = 0.41$; 20 mg/Kg, $p = 0.016$).

Figure 3: Effect of Therapeutic Oral WGP 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 Glucan (0, 22.6 $\pm$ 3.5, or 200.3 $\pm$ 36.4 μg/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 to a water control group. *p values were determined using a student's T-test (1.5 mg/Kg, p = 0.038; 13.3 mg/Kg, p = 0.01).

These cells are also necessary for efficient antigen presentation, and their level of activation can determine the effectiveness of the humoral (antibody) and cellular (T-cell) adaptive immune responses. The use of WGP glucan either in prophylaxis before exposure, and/or as part of a treatment regimen following exposure, represent two strategies that could provide clear health benefits whether the targeted population is the general public or the militaries exposed to a pathogenic challenge. Further, beta 1,3-glucan has clearly been shown to enhance the effectiveness of other medical countermeasures, such as vaccines and antibiotics [34-35]. The increase in survival time of mice prophylactically or therapeutically treated with WGP Glucan could also provide time for other anti-microbial therapies to be started and become effective.

The results of the studies reported in this communication provide clear preclinical "proof-of-concept" that a daily dose of WGP Glucan could provide a significant degree of protection against potential bioterroristic pathogenic agents, such as anthrax. Planned future work includes investigating the protective effects of WGP Glucan in a non-human primate model of anthrax infection, evaluating the combined prophylactic and therapeutic protective effect, defining the time of onset and duration of the protective effect, and evaluating synergy with antibiotic, antibody and vaccine therapies.

ACKNOWLEDGEMENT

The authors would like to thank all the individuals that made this work possible. A special acknowledgement to Dr. Lucie Richard, Anne Larrivée, Isabelle Lussier, Marie-Soleil Christin and Annie Venne from Biophage Pharma Inc. for their excellent work. We would like also to acknowledge Nicole Stady, Laurel Negrych and Dr. Jonh Cherwonogrodzky from DRES, for their invaluable support. This project was supported by DRDC, Defence Industrial Research (DIR), Biophage Pharma Inc., and Biopolymer Engineering, Inc.

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