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Enhanced clearance of a multiple antibiotic resistant *Staphylococcus aureus* in rats treated with PGG-glucan is associated with increased leukocyte counts and increased neutrophil oxidative burst activity

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Abstract

PGG-Glucan [Betafectin®], a highly purified soluble β-(1–6)-branched β-(1–3)-linked glucan isolated from *Saccharomyces cerevisiae*, has broad *in vitro* and *in vivo* anti-infective activities unrelated to cytokine induction. Here we present *in vivo* results on the anti-infective activity of PGG-Glucan against a multiple antibiotic resistant *Staphylococcus aureus*. PGG-Glucan (0.25–4 mg/kg) was administered intramuscularly to male Wistar rats 48 h, 24 h, and 4 h before and 4 h after intraperitoneal implantation of a gelatin capsule containing 10^8 *S. aureus* colony forming units (CFU). Blood samples were collected at various times after challenge to determine CFU levels, leukocyte counts and neutrophil oxidative burst activity; serum TNF-α and IL-1β levels were also evaluated. The 0.25 mg/kg PGG-Glucan dose had no effect on reducing blood CFU levels; however, PGG-Glucan doses of 0.5 mg/kg, 1 mg/kg, 2 mg/kg or 4 mg/kg significantly reduced blood CFU levels by 48 h after challenge. Reduced CFU levels correlated with significantly elevated absolute monocyte counts, absolute neutrophil counts, and neutrophil oxidative burst activity in the absence of any effect on TNF-α or on IL-1β levels. In additional studies, effects on mortality and blood CFU levels were evaluated in rats treated with ampicillin (an antibiotic to which the *S. aureus* was resistant), PGG-Glucan, or both agents. Mortality and blood CFU levels were reduced most in combination-treated rats compared to saline control rats or rats treated with either ampicillin alone or PGG-Glucan alone. We conclude that *in vivo* (1) PGG-Glucan can enhance clearance of an antibiotic resistant *S. aureus*, (2) that this clearance is accompanied by an increase in monocytes and neutrophils as well as a potentiation of neutrophil oxidative microbiocidal activity without alteration of the proinflammatory cytokine response, and (3) PGG-Glucan can enhance the effectiveness of traditional antibiotic treatment. © 1998 International Society for Immunopharmacology. Published by Elsevier Science Ltd

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1. Introduction

In recent years, infections caused by bacteria that are resistant to antibiotics have resulted in increased morbidity, mortality, and treatment costs (Neu, 1992; Tomasz, 1994; Service, 1995; Gold & Moellering, 1996). The rapid and continual emergence of antibiotic-resistant pathogens has created an urgent need for new antibacterial agents. Most new antibiotic agents under development are directed toward classical antimicrobial targets and are essentially improved versions of existing drugs (Service, 1995; Vaara, 1996). Unfortunately, introduction of new antimicrobials is almost always followed by emergence of pathogens resistant to the new agents (Neu 1992; Service, 1995; Gold & Moellering, 1996). It is clear that nonclassical approaches to the prevention and/or treatment of infections must be found. The use of immunomodulators to augment host defense responses is an alternative to the use of antibiotics in the prevention and/or treatment of infections caused by antibiotic-resistant pathogens.

Immunomodulators can be divided into two general classes based upon their biological source and their interaction with the immune system. One class consists of mammalian cell products (typically proteins) which selectively enhance activities of specific cells within the lymphohematopoietic system. Examples of such immunomodulators that are currently being clinically evaluated for the ability to treat and/or prevent infection are interferon gamma (IFN-γ), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Roilides et al., 1991; Farrar & Schreiber, 1993; Nemunaitis, 1993; Nelson, 1994; Aviles et al., 1996; Holyoake, 1996; Meropol et al., 1996; Murray, 1996). The second class of immunomodulators consists of substances isolated and purified from microorganisms or microbial culture fluids (Wooles & DiLuzio, 1963; Goren, 1982; Serrou & Rosenfeld, 1982; Yamamura & Kotani, 1982; Fudenberg et al., 1984; Dupont & Walker, 1993; Yamaguchi, 1993). These immunomodulators typically induce nonspecific stimulation of the lymphohematopoietic system, producing a broad spectrum of effects on many cell types. Until recently, the clinical use of this class of immunomodulators has been limited by their side effects, in particular, the induction of proinflammatory mediators.

β-(1–3) glucans are structurally complex, glucose homopolymers purified from fungi and yeast. A variety of these polysaccharides have been shown to bind to β-(1–3) glucan receptors on monocytes, macrophages, and neutrophils (Czop & Austen, 1985; Williams et al., 1986; Goldman, 1988; Muller et al., 1996; Thorton et al., 1996) and to induce the potentiation of a broad-spectrum of host defense responses (see reviews by Bleicher & Mackin, 1995; Williams et al., 1996). In preclinical animal models, a variety of β-(1–3) glucans have also been demonstrated to enhance resistance to challenge with various bacteria, viruses, fungi, and parasites (Williams et al., 1978; Reynolds et al., 1980; Cook et al., 1982; Kimura et al., 1983; Williams et al., 1983; Rasmussen et al., 1990; Pretus et al., 1991). However, because of the particulate or poorly soluble nature of most of these β-(1–3) glucans, and the propensity of these materials to induce proinflammatory mediators (Sherwood et al., 1987; Rasmussen et al., 1990; Rasmussen & Seljelid, 1991; Williams et al., 1996; Kilgore et al., 1997; Kulicke et al., 1997), these β-(1–3) glucans have had limited clinical use.

PGG-Glucan [Betafectin®] is a highly purified soluble β-(1–6)-branched β-(1–3)-linked glucan which is isolated from *Saccharomyces cerevisiae*. PGG-Glucan is completely soluble in aqueous solutions within the physiologic pH range and has a defined size range of $170{,}000 \pm 20{,}000$ daltons (Jamas et al., 1994; Bleicher & Mackin, 1995). This immunomodulator has been shown to have

broad *in vitro* and *in vivo* anti-infective activities (Lagrange & Fourgeaud, 1991; Onderdonk et al., 1992; Ostroff et al., 1993; Brunke-Reese & Mackin, 1994, Mackin et al., 1994a, Mackin et al., 1994b; Bleicher & Mackin, 1995; Mackin et al., 1995; Cisneros et al., 1996; Washburn et al., 1996; Tzianabos et al., 1998; Wakshull et al., 1998) without the induction of proinflammatory mediators (Poutsiaka et al., 1993; Mackin et al., 1994a; Mackin et al., 1994b; Bleicher & Mackin, 1995; Gibson et al., 1996; Adams et al., 1997; Patchen et al., 1998; Wakshull et al., 1997). In Phase II clinical trials, PGG-Glucan has been shown to reduce postoperative infection rates and to shorten the length of hospitalization (Babineau et al., 1994a; Babineau et al., 1994b). PGG-Glucan interacts with leukocytes through receptors that appear to be distinct from the receptors described for other β-(1–3) glucans (Michalek et al., 1998; Wakshull et al., 1996; Wakshull et al., 1998) and activates NF-κB and NF-IL-6 nuclear transcription factors (Adams et al., 1996; Pero et al., 1996; Adams et al., 1997). Although PGG-Glucan does not directly stimulate neutrophil function, it has been shown to prime neutrophils for activation by secondary stimuli (Mackin et al., 1994a; Mackin et al., 1994b; Bleicher & Mackin, 1995; Wakshull et al., 1998). This characteristic distinguishes PGG-Glucan from other β-(1–3) glucans which have been shown to directly activate cellular functions (Seljelid et al., 1981; Bogwald et al., 1982; Rasmussen & Seljelid, 1991; Williams et al., 1996).

Previous *in vivo* studies have shown that PGG-Glucan increases total leukocyte numbers, enhances clearance of bacteria from blood, and reduces mortality in rat intra-abdominal sepsis models (Onderdonk et al., 1992; Cisneros et al., 1996; Tzianabos et al., 1998). In the current studies, we have used a rat intra-abdominal infection model to evaluate the ability of PGG-Glucan to protect against a multiple antibiotic resistant bacteria and to further evaluate the mechanisms through which PGG-Glucan mediates its anti-infective activity. The data indicate that PGG-Glucan can enhance clearance of a multiple antibiotic resistant *S. aureus* and that this effect is accompanied by an increase in circulating monocytes and neutrophils as well as a potentiation of neutrophil oxidative microbicidal activity without an alteration of the proinflammatory cytokine response. In addition, PGG-Glucan synergizes with antibiotics to further enhance bacterial clearance and reduce infection-related mortality.

2. Experimental procedures

2.1. Animals

Virus- and antibody-free, male, Wistar rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed according to the National Institute of Health guidelines and were provided food and water *ad libitum*. Rats were quarantined for 7 days prior to being entered into experiments approved by the Alpha-Beta Technology Animal Care and Use Committee. Body weights of the rats ranged from 160–210 g at the time of experimentation.

2.2. Drug administration

PGG-Glucan (Betafectin® lot VB4-0001; Alpha-Beta Technology, Inc., Worcester, MA) was administered intramuscularly (i.m.) at doses of 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg or 4 mg/kg using a 25 gauge needle. Unless stated otherwise, PGG-Glucan was injected at 48 h, 24 h,

and 4 h before bacterial challenge and at 4 h after bacterial challenge. The endotoxin level in the PGG-Glucan preparation was less than 0.06 EU/mg. Ampicillin (Marsam Pharmaceuticals, Inc., Cherry Hill, NJ) was administered i.m. at a dose of 50 mg/kg at 6 h and 18 h after bacterial challenge.

2.3. Pharmacokinetic analysis

Groups of 4–5 rats were aseptically cannulated in the jugular vein 24 h prior to receiving 1 mg/kg of PGG-Glucan either as a single i.m. dose or as multiple i.m. doses given at 0 h, +24 h, +44 h, and +52 h (spacing of dosing times equivalent to the −48 h, −24 h, −4 h, +4 h PGG-Glucan treatment regimen used in most of our *S. aureus* infection studies). At each sample time, approximately 250 μl of blood was collected from each rat *via* the jugular cannula. In the single-dose group, blood samples were collected before PGG-Glucan dosing and at 0.08 h, 0.25 h, 0.50 h, 0.75 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h and 48 h after PGG-Glucan dosing. In the multiple-dose group, blood samples were collected immediately before the last PGG-Glucan dose (+52 h), and at 0.08 h, 0.25 h, 0.50 h, 0.75 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, and 72 h after the last PGG-Glucan dose. Blood samples were placed into sterile tubes containing acid-citrate-dextrose (ACD) and stored on ice until plasma was isolated; plasma was then frozen at −40°C until analyzed. Plasma PGG-Glucan concentrations were determined using a chromogenic *Limulus* amebocyte lysate assay (Tanaka et al., 1991). A 1:50 dilution of plasma in sterile water was heat-inactivated (56°C, 30 min) and denatured for 30 min in 1 N NaOH at room temp. Samples were neutralized with Tris-HCl and quantified against known concentrations of PGG-Glucan spiked into normal control rat plasma. Pyrochrome™ lysate/Tris buffer reagent (50 μl; Associates of Cape Cod, Falmouth, MA) was added to 50 μl of each diluted plasma sample in pyrogen-free 96 well microtiter plates. The samples were incubated at 37°C for a time depending on lysate strength, and the reaction stopped with acetic acid. PGG-Glucan concentrations were determined based on sample absorbance at 405 nm using Softmax Pro software for analysis (volume 1.1; Molecular Devices, Melo Park, CA). Pharmacokinetic analyses of plasma PGG-Glucan concentrations *vs* time were performed according to standard procedures (Gibaldi & Perrier, 1982; Shargel & Yu, 1985). A comparison between one and two compartment nonlinear fits of single and multiple dose data showed best agreement with a one compartment model. The absorption phase was fit through feathering. Area under the plasma concentration *vs* time curve (AUC) was determined *via* trapezoidal rule, with extrapolation to infinity ($AUC_{0-\infty}$). Maximum drug concentration values (C_{max}) and time values (t_{max}) were determined through inspection. Clearance (CL) and steady state volume of distribution (V_d) were calculated according to standard procedures (Gibaldi & Perrier, 1982).

2.4. Bacteria

A multiple antibiotic resistant *S. aureus*, originally isolated from a clinical wound site, was kindly provided by Dr Andrew B. Onderdonk (Channing Laboratory, Harvard Medical School, Boston, MA). The antibiotic sensitivity of this *S. aureus* is shown in Table 1. Bacteria were expanded in Trypticase Soy Broth (TSB; Becton Dickinson Microbiology Systems, Cockeysville, MD) for 18 h at 37°C and stock aliquots frozen at −80°C until used. To determine the viability

Table 1.
S. aureus antibiotic sensitivity[a,b]

Sensitive[c]	Intermediate[c]	Resistant[c]
Gentamicin	Chloramphenicol	Ampicillin
Tetracycline	Streptomycin	Cephalothin
Tribrissen		Ciprofloxacin
Vancomycin		Clindamycin
		Erythromycin
		Kanamycin
		Oxacillin
		Penicillin-G

[a] *S. aureus* #819482 provided by Dr Andrew B. Onderdonk, Harvard Medical School, Boston, MA.
[b] Analyzed using a Vitek System.
[c] Based on Kirby–Bauer sensitivities as defined by The National Committee for Clinical Laboratory Science.

of stock bacteria, a frozen stock sample was thawed, serially diluted, and 50 μl samples plated onto Trypticase Soy Blood Agar plates (TSBA; Becton Dickinson Microbiology Systems, Cockeysville, MD). Plates were cultured for 48 h at 37°C, the bacterial colonies were counted, and the number of bacterial colony forming units (CFU) per ml of stock was calculated. To prepare bacterial inoculum for *in vivo* studies, stock cultures were diluted to the desired number of CFU/ml in Phosphate Buffered Saline (PBS; Gibco Life Technologies, Grand Island, NY) containing 1% dextran sulfate and a final concentration of 5% barium sulfate (wt/vol). A 0.5 ml aliquot of the appropriately diluted bacteria was aseptically placed into 2 cm long $\times$ 0.5 cm diameter gelatin capsules (Eli-Lilly Inc., Indianapolis, IN) and the capsules were implanted into the peritoneal cavity of rats as described below.

2.5. Surgery for capsule implantation

Rats were anesthetized by i.m. injection of a mixed anesthetic cocktail consisting of Ketamine (Fort Dodge Laboratoires, Inc., Fort Dodge, IA), PromAce (Ayerst Laboratories, Inc., Rouses Point, NY), Xylazine (Phoenix Scientific, Inc., St. Joseph, MO) and saline (750 mg, 10 mg, 100 mg, saline to 20 ml, respectively) using a 25 gauge needle. The anesthesia was adjusted for each rat based on body weight by administering 0.0019 ml/g body weight. After administering the anesthesia, the abdomen of each rat was shaved, cleaned with iodine solution, and a 1.5 cm anterior midline incision was made through the abdominal wall and the peritoneum. The gelatin capsule containing a specified inoculum of *S. aureus* CFU was immediately placed into the peritoneal cavity and the incision was closed with interrupted 3–0 silk sutures. The total duration of surgery was less than 2 min. Following surgery, animals were housed five per cage and monitored every 15 min for the first 2 h, every 30 min for the next hour, and then three times per day for the duration of the experiment.

2.6. Blood sampling and analysis

Animals were anesthetized with O_2:CO_2 (1:1) and 2 ml of blood was obtained by cardiac puncture using a 3 ml syringe with 20 gauge needle. Immediately after blood was collected, ~1.5 ml was expelled into a 1.7 ml micro-centrifuge tube (Corning Costar Corporation, Cambridge, MA) containing 5 units of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ). Each animal was then humanely euthanized with CO_2. For each blood sample, 20 ml of 50°C Tryptic Soy Agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) was plated into a sterile petri plate and a 0.5 ml aliquot of blood immediately added to the plate and thoroughly mixed into the agar by swirling the plate. Once the agar solidified, plates were incubated at 37°C for 48 h, then colonies were enumerated. Blood CFU data are expressed as log CFU/ml of blood. The remaining blood from each sample was used for assessment of total and differential cell counts. Total white blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts were performed on a System 9010+ Hematology Analyzer (Biochem Immunosystems, Inc., Allentown, PA). For WBC differential analysis, smears of each blood sample were prepared and stained with Diff–Quik (Dade Diagnostics, Inc., Aguada, PR). The percentages of neutrophils, monocytes and lymphocytes were used to calculate absolute neutrophil counts, absolute monocyte counts, and absolute lymphocyte counts.

2.7. Neutrophil separation and oxidative burst assay

Polymorphonuclear neutrophils (PMNs) were isolated at room temperature, using NIM 2 (Cardinal Associates, Inc., Santa Fe, NM). A 2.5 ml volume of NIM 2A (lower gradient) was placed into a 15 ml polypropylene tube and 2.5 ml of NIM 2B (upper gradient) was carefully layered above it. Approximately 2.5 ml of rat blood was layered onto the upper gradient and the tubes were centrifuged at $900 \times g$ for 45 min with the brake off. From each tube, the plasma and upper band (containing monocytes) were removed and the lower band (containing the PMNs) was collected and placed into a fresh tube containing 5 ml of calcium- and magnesium-free Hanks' Balanced Salt Solution containing 10 mM Hepes (HBSS/Hepes). The tubes were mixed thoroughly, centrifuged at $400 \times g$ for 10 min and the supernatants discarded. Contaminating red blood cells were hypotonically lysed by a 15 s incubation with 27 ml of deionized water (dH_2O). Then 9 ml of 3.6% NaCl was immediately added and the tubes were centrifuged at $400 \times g$ for 5 min. The supernatants were discarded and the remaining PMNs were resuspended in 5 ml of HBSS/Hepes. Cell counts were performed on a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) and the purity of the PMN samples assessed in Diff–Quik (Dade Diagnostics, Inc., Aguada, PR) stained cytospin preparations. Cell preparations were found to contain greater than 95% PMNs. For the oxidative burst assay, cell suspensions were centrifuged at $400 \times g$ for 7 min and resuspended at 2.5×10^6 cells/ml in HBSS containing 50 μM Luminol (Sigma Chemical Co., St Louis, MO). A 100 μl volume of each cell suspension was placed into triplicate wells of 96-well plates. The plates were covered and incubated at 37°C for 5–10 min prior to the addition of 100 μl of phorbol 12-myristate-13-acetate (PMA) at a final concentration of 48 ng/ml. Plates were immediately placed in a 37°C Microtiter Plate Luminometer (Dynatech Laboratoires, Chantilly, VA) and read for 80 cycles with a 15 s pause between cycles (~90 min total time). Data for PMN oxidative burst activities are expressed as chemiluminescence activities (CLA) which were determined by measuring

Table 2
Pharmacokinetic parameters for PGG-Glucan following intramuscular administration in rats

Absorption $t_{1/2}$ (h)	Elimination $t_{1/2}$ (h)	C_{max} (μg/ml)	t_{max} (h)	$AUC_{0-\infty}$ (μg.h/ml)	CL[b] (ml/h)	V_d[c] (ml)
Single Intramuscular Dose[a]						
0.72 ± 0.20	3.74 ± 0.64	0.44 ± 0.11	1.15 ± 0.37	2.45 ± 0.79	41 ± 13	217 ± 53
Multiple Intramuscular Dose[d]						
0.69 ± 0.14	3.02 ± 0.22	0.50 ± 0.14	1.22 ± 0.19	2.66 ± 0.65	42 ± 11	182 ± 54

[a] One-compartment model; Mean ± SEM of data obtained from 5 rats.
[b] $CL = (F \times Dose)/AUC_{0-\infty}$(13).
[c] $V_d = (F \times Dose)/(K \times AUC_{0-\infty})$ (13).
[d] One-compartment model; Mean ± SEM of data obtained from 4 rats.

the area under the chemiluminescence intensity curves using commercial software (Dynatech Laboratories, Chantilly, VA).

2.8. Cytokine analysis

Serum samples for cytokine analysis were collected at various times after bacterial challenge in rats administered 0.5 mg/kg of PGG-Glucan at 48 h, 24 h, and 4 h before and at 4 h after challenge. To obtain positive control sera, rats were intravenously (i.v.) administered lipopolysaccharide (LPS; *E. coli* 0127:B8; Sigma Chemical Co., St Louis, MO) at a dose of 10 mg/kg and serum samples collected at 2 h after LPS injection. Rat cytokines were quantitated by ELISA using commercial kits for detection of TNF-α (Genzyme Corporation, Cambridge, MA) and IL-1β (Endogen, Inc., Woburn, MA).

2.9. Statistical analysis

Unless indicated otherwise, results are expressed as the mean ± standard error of the mean (SEM) of data obtained from replicate experiments. All data, with the exception of mortality data, were analyzed by unpaired Student t-tests using EXCEL 5.0 software (Microsoft Corporation, Redmond, WA); mortality data were analyzed by Fisher exact tests using the same software. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Pharmacokinetic studies

Initial studies evaluated the bioavailability of PGG-Glucan when administered i.m. (Table 2). The absorption phase half-life ($t_{1/2}$) following a single i.m. PGG-Glucan administration (0.72 ± 0.20 h) was roughly 5-fold less than the terminal elimination phase half-life (3.74 ± 0.64 h), indicating

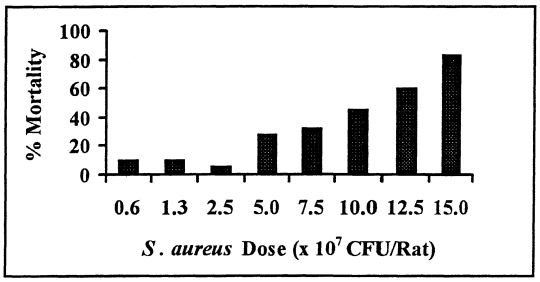

Fig. 1. Mortality of male Wistar rats challenged with various doses of *S. aureus*. Groups of 20–35 rats were administered the indicated doses of bacteria and mortality monitored over a five day observation period. Data represent composite results from 2–3 experiments.

facile diffusion into the systemic circulation. The maximum plasma PGG-Glucan level (0.44 ± 0.11 μg/ml) was obtained approximately 1 h after dosing. $AUC_{0-\infty}$ was 2.45 ± 0.79 μg hr/ml, with a *CL* value of 41 ± 13 ml/hr and a V_d value of 217 ± 53 ml. Following administration of multiple PGG-Glucan doses, no measurable accumulation of PGG-Glucan was discernible. This was evidenced by almost identical pharmacokinetic profiles in animals receiving single *vs* multiple doses of PGG-Glucan.

3.2. Bacterial dose response

Initial studies established the virulence of the *S. aureus* chosen for our studies. Rats were challenged with various doses of *S. aureus* and mortality was monitored over a five day observation period. Figure 1 illustrates that the *S. aureus* induced a dose-related mortality, with less than 10% mortality at *S. aureus* doses of 0.625×10^7, 1.25×10^7, and 2.5×10^7 CFU/rat, and 28%, 32%, 45%, 60% and 83% mortality at *S. aureus* doses of 5.0×10^7, 7.5×10^7, 10.0×10^7, 12.5×10^7 and 15.0×10^7 CFU/rat, respectively. Among the animals that succumbed, approximately 90% did so within 48 h after bacterial challenge. To determine the minimally lethal bacterial dose conducive to monitoring sustained and accurately quantifiable blood CFU levels (i.e., 40–400 or log 1.6–2.6 CFU/ml blood), rats were challenged with *S. aureus* doses of 2.5×10^7, 5×10^7 and 10×10^7 CFU/rat and blood CFU levels were determined at 6 h, 24 h, 36 h and 48 h after challenge. *S. aureus* doses of 2.5×10^7 and 5×10^7 CFU/rat produced only a transient increase in blood CFU levels (Table 3). However, increasing the *S. aureus* dose to 10×10^7 CFU/rat consistently resulted in mean blood CFU levels greater than 40/ml for up to 48 h after challenge. Based on these results, the *S. aureus* dose of 10×10^7 CFU/rat was chosen for subsequent experiments. This challenge dose was consistently associated with approximately 50% mortality within 48 h after challenge.

3.3. Effects of PGG-Glucan on blood CFU levels

Previous studies had shown that PGG-Glucan at the dose of 0.5 mg/kg, when administered 48 h, 24 h and 4 h before and 4 h after challenge, could enhance survival and reduce blood CFU levels in an intra-abdominal *E. coli* rat sepsis model (Onderdonk et al., 1992; Cisneros et al., 1996). Thus, this PGG-Glucan dose and treatment regimen were initially chosen to evaluate the anti-

Table 3
Detection of blood CFU levels in rats challenged with *S. aureus*[a]

Time after challenge (h)	2.5×10^7 CFU/rat	5×10^7 CFU/rat	10×10^7 CFU/rat
6	3.00 ± 0.24 (2/9)[b]	3.50 ± 0.21 (1/4)	3.93 ± 0.08 (2/22)
24	1.78 ± 0.18 (2/9)	2.04 ± 0.18 (1/3)	2.26 ± 0.11 (3/16)
36	NA[c]	1.77 ± 0.14 (1/5)	2.12 ± 0.23 (2/11)
48	0.82 ± 0.24 (2/8)	1.27 ± 0.20 (4/25)	1.63 ± 0.16 (4/33)

[a] Log CFU/ml blood; Mean ± SEM of data obtained from all experiments.
[b] Number of experiments/Total number of rats evaluated.
[c] Not analyzed.

Table 4
Effect of PGG-Glucan treatment on blood CFU levels in rats challenged with 10^8 *S. aureus*[a]

	Log CFU/ml blood[b]		
Time after challenge (h)	PGG-Glucan	Saline	*P* value[c]
6	3.77 ± 0.11 (3/27)[d]	3.97 ± 0.07 (3.27)	NS[e]
24	2.31 ± 0.09 (4/22)	2.39 ± 0.11 (4/22)	NS
48	0.89 ± 0.11 (4/40)	1.66 ± 0.14 (4/37)	<0.001
72	0.60 ± 0.13 (3/35)	1.19 ± 0.17 (3/35)	<0.01

[a] Rats injected i.m. with either saline or PGG-Glucan (0.5 mg/kg) at 48 h, 24 h, and 4 h before challenge and 4 h after challenge.
[b] Mean ± SEM of data obtained from all experiments.
[c] *t*-test; PGG-Glucan *vs* saline.
[d] Number of experiments/Total number of rats evaluated.
[e] Not statistically significant.

infective effects of PGG-Glucan in our antibiotic resistant *S. aureus* model. Rats were administered PGG-Glucan and blood CFU levels were determined at various times after challenge with 10^8 *S. aureus* CFU. As can be seen in Table 4, rats treated with PGG-Glucan exhibited significantly lower blood CFU levels than saline-treated rats at 48 h ($P < 0.001$) and at 72 h ($P < 0.01$) after challenge.

The anti-infective potentials of alternate PGG-Glucan treatment regimens were also evaluated. In all regimens, the 0.5 mg/kg PGG-Glucan dose was used and blood CFU levels were evaluated at 48 h after *S. aureus* challenge. Data in Table 5 illustrate that, while rats treated with the −48 h, −24 h, −4 h, +4 h PGG-Glucan regimen exhibited the greatest reduction in blood CFU levels, blood CFU levels also appeared to be slightly reduced in rats treated with the −24 h, −4 h, +4 h and the −24 h, +4 hr PGG-Glucan regimens; however, these CFU levels were not statistically different from control levels. Other treatment regimens appeared to be ineffective in reducing blood CFU levels.

To further characterize the anti-infective activity of PGG-Glucan, PGG-Glucan dose response

Table 5
Effect of PGG-Glucan treatment regimens on blood CFU levels in rats 48 h after challenge with 10^8 *S. aureus*

	Log CFU/ml blood[b]	
Regimen[a]	PGG-Glucan	Saline
−48, −24, −4, +4 h	1.42 ± 0.11[c] (5/62)[d]	1.82 ± 0.12 (5/62)
−24, −4, +4 h	1.46 ± 0.21 (2/24)	1.86 ± 0.22 (2/21)
−4, +4 h	1.44 ± 0.26 (1/15)	1.49 ± 0.21 (1/14)
−24, +4 h	2.05 ± 0.23 (2/14)	2.36 ± 0.21 (2/20)
−24, −4 h	1.69 ± 0.15 (3/41)	1.79 ± 0.14 (3/41)

[a] Rats injected i.m. with either saline or PGG-glucan (0.5 mg/kg) at the indicated hours with respect to *S. aureus* challenge.
[b] Mean ± SEM of data obtained from all experiments.
[c] *t*-test; $P < 0.05$ *vs* saline.
[d] Number of experiments/Total number of rats evaluated.

Table 6
Effect of PGG-Glucan dose on blood CFU levels in rats 48 h after challenge with 10^8 *S. aureus*

Treatment[a]	Log CFU/ml blood[b]	*P* value[c]
Saline	1.70 ± 0.13 (4/35)[d]	
PGG-Glucan (mg/kg)		
0.25	1.66 ± 0.29 (1/8)	NS[e]
0.5	0.99 ± 0.17 (2/17)	< 0.01
1	1.12 ± 0.12 (3/32)	< 0.01
2	0.97 ± 0.15 (2/21)	< 0.001
4	1.06 ± 0.20 (1/9)	< 0.05

[a] Rats injected i.m. with either saline or PGG-Glucan at 48 h, 24 h, and 4 h before challenge and 4 h after challenge.
[b] Mean ± SEM of data obtained from all experiments.
[c] *t*-test; PGG-Glucan *vs* saline.
[d] Number of experiments/Total number of rats evaluated.
[e] Not statistically significant.

experiments were conducted. PGG-Glucan at doses ranging from 0.25–4 mg/kg were administered to rats at 48 h, 24 h and 4 h before challenge and at 4 h after challenge with 10^8 *S. aureus* CFU. Since initial experiments had indicated that a reduction in blood CFU levels in PGG-Glucan-treated rats was first evident at 48 h after challenge (Table 4), blood CFU levels were evaluated at the 48 h time point. The data in Table 6 indicate that a PGG-Glucan dose of 0.25 mg/kg had no effect on reducing blood CFU levels. However, PGG-Glucan at doses of 0.5 mg/kg, 1 mg/kg, 2

Table 7
Effect of PGG-Glucan on peripheral blood cell counts in rats before and after challenge with 10^8 *S. aureus*[a]

	WBC ($\times 10^6$/ml)[b]		RBC ($\times 10^9$/ml)		PLT ($\times 10^6$/ml)	
Time	Saline	PGG-Glucan	Saline	PGG-Glucan	Saline	PGG-Glucan
Prechallenge						
−48 h (Baseline)	12.9±0.6	12.9±0.6	5.92±0.12	5.92±0.12	592±67	592±67
−24 h	14.5±1.0	13.5±1.4	6.31±0.18	6.38±0.11	317±57	445±55
−4 h	13.8±1.1	12.2±0.8	6.16±0.23	6.25±0.24	376±46	511±53[d]
0 h	13.0±1.0	12.9±0.9	6.13±0.14	6.09±0.16	444±97	583±75
Postchallenge						
6 h	18.6±1.1	20.6±1.9 (11%)[c]	6.15±0.15	6.20±0.13	858±33	768±93
24 h	9.4±0.6	10.1±1.1 (7%)	6.88±0.28	6.99±0.22	756±47	807±49
48 h	11.2±0.6	14.0±0.7 (25%)[d]	6.08±0.09	6.09±0.10	704±35	620±49
72 h	15.2±1.3	16.0±1.6 (5%)	6.36±0.12	6.58±0.23	600±98	611±57

[a] Rats injected i.m. with either saline or PGG-Glucan (0.5 mg/kg) at 48 h, 24 h, and 4 h before and 4 h after challenge.
[b] Mean±SEM of 10 blood samples at all time points except 48 h postchallenge where 35 saline and 29 PGG-Glucan samples were analyzed and 72 h postchallenge where 11 saline and 13 PGG-Glucan samples were analyzed.
[c] Percent change *vs* saline.
[d] *t*-test; $P < 0.05$ *vs* saline.

mg/kg and 4 mg/kg did significantly reduce blood *S. aureus* numbers compared to saline treatment. The CFU reduction observed in rats treated with PGG-Glucan was a threshold response, with similar effects being observed at all effective PGG-Glucan doses.

3.5. *Effect of PGG-Glucan on peripheral blood cell counts*

To evaluate whether the anti-infective activity of PGG-Glucan was related to an ability to increase WBC numbers, blood cell counts were performed at various times following a 10^8 *S. aureus* CFU challenge in rats treated with 0.5 mg/kg of PGG-Glucan. Since rats received three PGG-Glucan injections prior to infectious challenge in the −48 h, −24 h, −4 h and +4 h treatment regimen, blood cell counts were evaluated during the prechallenge treatment period as well as during the postchallenge period (Tables 7 and 8). During the prechallenge period (i.e., after one, two, or three doses of PGG-Glucan, but prior to infectious challenge) WBC counts were not altered compared to either baseline values or to values in rats injected with saline (Table 7). Following *S. aureus* challenge, WBC counts increased in all animals at 6 h, dropped at 24 h, and increased again at 48 h and 72 h (Table 7). At all postchallenge time points, WBC counts in rats treated with PGG-Glucan were maintained at higher levels than in saline-treated rats. However, a statistical difference was observed only at the 48 h time point. Differential analysis revealed that the increased WBC numbers observed in rats treated with PGG-Glucan were attributed to increases in both monocytes and neutrophils as reflected by the 77–125% increase in absolute monocyte numbers and the 19–38% increase in absolute neutrophil numbers (Table 8). Lymphocyte values

Table 8. Effect of PGG-Glucan on peripheral blood cell differential counts in rats before and after challenge with 10^8 *S. aureus*[a]

	Neutrophils ($\times 10^6$/ml)[b]		Monocytes ($\times 10^6$/ml)		Lymphocytes ($\times 10^6$/ml)	
Time	Saline	PGG-Glucan	Saline	PGG-Glucan	Saline	PGG-Glucan
Prechallenge						
−48 h (Baseline)	1.7±0.2	1.7±0.2	0.09±0.02	0.09±0.02	10.1±0.5	10.1±0.5
−24 h	1.4±0.2	2.0±0.4	0.17±0.07	0.25±0.04	12.9±0.9	11.2±1.11
−4 h	1.6±0.3	1.4±0.2	0.19±0.05	0.17±0.07	11.8±0.9	10.5±0.8
0 h	1.9±0.2	1.6±0.2	0.10±0.03	0.08±0.03	10.9±0.9	11.1±0.8
Postchallenge						
6 h	2.8±0.4	2.1±0.2 (−25%)[c]	0.18±0.06	0.24±0.04 (33%)	15.6±1.0	18.1±1.9 (16%)
24 h	3.2±0.3	3.8±0.5 (19%)	0.12±0.03	0.27±0.04 (125%)[d]	6.1±0.4	5.9±0.6 (−3%)
48 h	2.9±0.2	4.1±0.3 (38%)[d]	0.13±0.02	0.23±0.04 (77%)[d]	8.3±0.5	9.8±0.5 (19%)[d]
72 h	3.6±0.4	4.9±0.6 (36%)[d]	0.19±0.04	0.56±0.13 (95%)[d]	11.2±1.1	10.4±1.0 (−7%)

[a] Rats injected i.m. with either saline or PGG-Glucan (0.5 mg/kg) at 48 h, 24 h and 4 h before and 4 h after challenge.
[b] Mean±SEM of 10 blood samples at all time points except 48 h postchallenge where 35 saline and 29 PGG-Glucan samples were analyzed and 72 h postchallenge where 11 saline and 13 PGG-Glucan samples were analyzed.
[c] Percent change *vs* saline.
[d] *t*-test; $P < 0.05$ *vs* saline.

in rats treated with PGG-Glucan were also maintained at a higher level than in saline-treated rats at 48 h after challenge (Table 8). Even at 72 h after challenge, absolute monocyte and absolute neutrophil counts were significantly elevated in PGG-Glucan-treated rats compared to saline-treated rats. Although PLT values dropped slightly in all prechallenge rats following either saline or PGG-Glucan administration, at the −24 h, −4 h and 0 h time points, PLT values in PGG-Glucan-treated rats were higher than in saline-treated rats; this pattern, however, did not persist after *S. aureus* challenge (Table 7). PGG-Glucan did not change RBC values either before or after bacterial challenge (Table 7).

3.6. Effect of PGG-Glucan on neutrophil oxidative burst activity

To evaluate whether PGG-Glucan treatment altered neutrophil oxidative microbicidal activity, rats were injected with 0.5 mg/kg of PGG-Glucan 48 h, 24 h, and 4 h before and 4 h after a 10^8 *S. aureus* CFU challenge and PMNs evaluated at various times for oxidative burst activity. As can be seen in Fig. 2, PGG-Glucan treatment had no effect on PMN oxidative burst activity prior to *S. aureus* challenge. However, PMN oxidative burst activity was significantly increased in PMNs obtained from PGG-Glucan-treated rats at 48 h ($P < 0.05$) and at 72 h ($P < 0.01$) after challenge.

3.7 Effect of PGG-Glucan on cytokine release

Agents capable of altering the proinflammatory cytokine response have previously been shown to possess anti-infective activity (Dinarello, 1994; Amura et al., 1995). Because of this, the ability

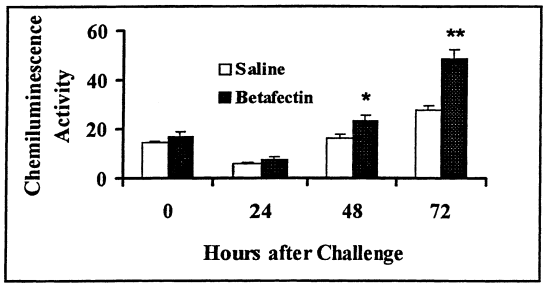

Fig. 2. Effect of PGG-Glucan treatment (0.5 mg/kg at 48 h, 24 h and 4 h before challenge and 4 h after challenge) on PMN oxidative burst activity in male Wistar rats challenged with 10^8 *S. aureus*. Data represent the mean ± SEM of results obtained in 2 experiments, each evaluating 5 rats per group. * $P < 0.05$ *vs* saline; ** $P < 0.01$ *vs* saline.

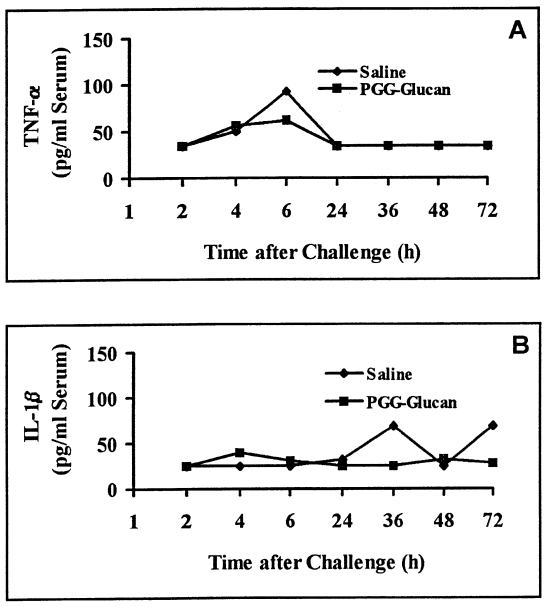

Fig. 3. Effect of PGG-Glucan treatment on TNF-α (A) and IL-1β (B) induction in male Wistar rats challenged with 10^8 *S. aureus*. Data represent the mean ± SEM of results obtained in 2 experiments, each evaluating 5 rats per group. In the same assays, positive control sera obtained from 5 rats 2 h after i.v. administration of 10 mg/kg of LPS exhibited 4922 ± 581 pg/ml of TNF-α and 1065 ± 80 pg/ml of IL-1β.

of PGG-Glucan to alter proinflammatory cytokine release was examined. Rats were injected with 0.5 mg/kg of PGG-Glucan 48 h, 24 h, and 4 h before and 4 h after a 10^8 *S. aureus* CFU challenge and serum samples collected at various times thereafter. The data presented in Fig. 3 illustrate that, compared to saline treatment, PGG-Glucan treatment altered neither TNF-α nor IL-1β levels in *S. aureus* infected rats.

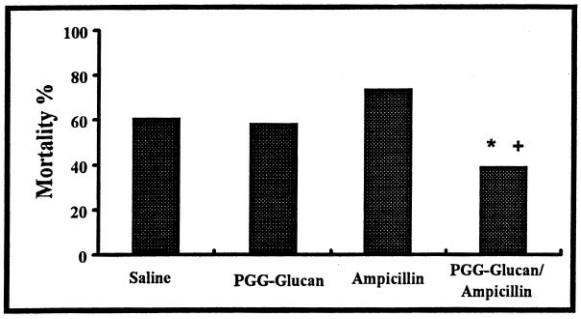

Fig. 4. Effect of PGG-Glucan and ampicillin on mortality in male Wistar rats challenged with 1.25×10^8 *S. aureus*. Rats were i.m. administered PGG-Glucan (0.5 mg/kg at 48 h, 24 h, and 4 h before and 4 h after bacterial challenge), ampicillin (50 mg/kg at 6 h and 18 h after bacterial challenge), or both PGG-Glucan and ampicillin. Mortality was then monitored over a 48 h period. Data represent composite results from 2 experiments, each evaluating 25 rats per group. * $P < 0.05$ *vs* saline. + $P < 0.05$ *vs* ampicillin or PGG-Glucan.

3.8. Effects of combined ampicillin and PGG-Glucan treatment on mortality and CFU levels

Additional studies assessed the anti-infective effects of PGG-Glucan when used in combination with antibiotics in rats infected with *S. aureus*. Rats were administered PGG-Glucan or/and ampicillin, an antibiotic to which the *S. aureus* used in these studies exhibited strong resistance (Table 1). Animals were then challenged with 1.25×10^8 *S. aureus* CFU and mortality monitored over a 48 h period. A slightly higher (i.e., more lethal) *S. aureus* challenge dose was intentionally used in these studies to facilitate the ability to observe a reduction in mortality in combination-treated rats. It can be seen in Fig. 4 that, while neither PGG-Glucan nor ampicillin alone altered *S. aureus*-induced mortality, significantly reduced mortality was observed in rats treated with the combination of these agents. Additional studies evaluated CFU levels in rats challenged with *S. aureus* CFU (Table 9). At 48 h postchallenge, PGG-Glucan treatment alone was able to slightly reduce blood CFU levels in *S. aureus*-infected rats while ampicillin treatment alone had no effect on CFU numbers. In combination-treated rats, however, CFU numbers were reduced significantly further than in rats treated with only PGG-Glucan.

4. Discussion

In recent years, the emergence of numerous antibiotic-resistant bacterial pathogens has led to an urgent need for new antibacterial agents (Neu, 1992; Tomasz, 1994; Service, 1995; Gold & Moellering, 1996). Augmentation of host defense responses by immunomodulators is an alternative to the use of antibiotics in the prevention and/or treatment of infections caused by antibiotic resistant bacteria.

It has been shown that the immunomodulator PGG-Glucan induces anti-infective activity. *In vitro*, PGG-Glucan can selectively enhance the microbicidal activities of neutrophils and macrophages without stimulating proinflammatory cytokine production (Ostroff et al., 1993; Poutsiaka et al., 1993; Brunke-Reese & Mackin, 1994; Mackin et al., 1994a; Mackin et al., 1994b; Bleicher

Table 9
Effect of PGG-Glucan and ampicillin on blood CFU levels in rats 48 h after challenge with *S. aureus*

Treatment[a]	Log CFU/ml blood[b]
Saline	1.99 ± 0.27 (2/31)[c]
PGG-Glucan	1.55 ± 0.25 (2/22)
Ampicillin	2.17 ± 0.27 (2/23)
PGG-Glucan + ampicillin	0.81 ± 0.16 (2/31)[d,e,f]

[a] Rats injected i.m. with either saline, 0.5 mg/kg of PGG-Glucan at 48 h, 24 h, and 4 h before challenge and 4 h after challenge, 50 mg/kg of ampicillin at 6 h and 18 h after challenge, or with both PGG-Glucan and ampicillin.
[b] Mean ± SEM of data obtained from all experiments.
[c] Number of experiments/Total number of rats evaluated.
[d] *t*-test; $P < 0.05$ *vs* saline.
[e] *t*-test; $P < 0.05$ *vs* PGG-Glucan alone.
[f] *t*-test; $P < 0.05$ *vs* ampicillin alone.

& Mackin, 1995; Mackin et al., 1995; Gibson et al., 1996; Washburn et al., 1996; Adams et al., 1997; Patchen et al., 1998; Wakshull et al., 1997). Interestingly, these data indicate that PGG-Glucan has no direct effect on neutrophil or monocyte functions but rather primes neutrophils and monocytes for more robust responses when exposed to a second stimulus such as PMA, fMLP, or opsonized bacteria (Brunke-Reese & Mackin, 1994; Mackin et al., 1994a; Mackin et al., 1994b; Bleicher & Mackin, 1995; Mackin et al., 1995; Wakshull et al., 1997). PGG-Glucan has also been demonstrated to possess *in vivo* anti-infective activity. In rodent models of gram negative and mixed microbial infections, PGG-Glucan treatment has been demonstrated to reduce mortality and to enhance bacterial clearance (Onderdonk et al., 1992; Stashenko et al., 1995; Cisneros et al., 1996; Tzianabos et al., 1998). The *in vivo* data presented in this report (Tables 4–6) further demonstrate the ability of PGG-Glucan to protect against bacterial infection, specifically infection caused by a multiple antibiotic resistant gram positive organism (Table 1).

Studies evaluating combination treatment with PGG-Glucan and ampicillin further illustrated the anti-infective potential of PGG-Glucan (Fig. 4 and Table 9). These studies demonstrated that, when PGG-Glucan was used in combination with an antibiotic to which the *S. aureus* exhibited high resistance, significantly reduced mortality and CFU levels were observed compared to either treatment alone. With respect to this synergism, it is speculated that although the antibiotic alone was ineffective at killing the *S. aureus*, it apparently could somehow impair these organisms such that they were more easily killed by the PGG-Glucan-primed leukocytes. A similar synergistic phenomenon has also recently been noted by Tzianabos *et al.* (1996). In these studies, they observed synergistic survival enhancement with PGG-Glucan and cephalothin or gentamicin in *E. coli*-infected rats and with PGG-Glucan and ciprofloxacin in *S. aureus*-infected rats.

Primarily prophylactic dose regimens have been evaluated in previous *in vivo* PGG-Glucan studies (Onderdonk et al., 1992; Stashenko et al., 1995; Cisneros et al., 1996; Tzianabos et al.,

1996). Although most studies in this report also focused on effects produced by a primarily prophylactic PGG-Glucan treatment regimen (−48 h, −24 h, −4 h, +4 h), several other treatment regimens were evaluated (Table 5). While the above treatment regimen produced the best results, the −24 h, −4 h, +4 h and the −24 h, +4 hr treatment regimens also appeared to slightly enhance CFU clearance in *S. aureus*-infected rats. Given the observation that all regimens including PGG-Glucan administration at both −24 h and +4 h somewhat reduced blood CFU levels stresses the importance of a multiple dose treatment regimen. However, not only did repeat exposure to PGG-Glucan appear to be is necessary for optimal biological activity, but the timing of exposure was also critical.

The reason why some of the treatment regimens evaluated were ineffective is not known. In all the treatment regimens, PGG-Glucan was administered i.m. Based on the 3.74 ± 0.64 h terminal half-life of i.m. administered PGG-Glucan, it can be calculated that ~99% of the drug would be eliminated within ~22 h (6 half-lives) after administration (Wingard et al., 1991). It is especially perplexing that the −4 h, +4 h treatment regimen was ineffective, since in this regimen there was only an 8 h separation between PGG-Glucan doses and more drug would be present to induce a biological response at the time of infection than, for example, in the −24 h, +4 h regimen which was effective. The i.m. absorption phase half-life of 0.72 ± 0.20 h indicated that drug was entering the circulation at a rapid rate relative to the terminal elimination phase half-life of 3.74 ± 0.64 h. The V_d value observed following i.m. PGG-Glucan administration (217 ± 53 ml) was greater than previously observed following i.v. PGG-Glucan administration (63 ± 14 ml; Gerard Palace, Alpha-Beta Technology, Inc., personal communication), indicating that i.m. administered PGG-Glucan initially distributes within the muscle peripheral compartment and subsequently diffuses towards the central blood compartment. This may be important for immunomodulatory drugs whose target cells may reside in both the periphery (e.g., tissue neutrophils, macrophages) and the circulation (e.g., circulating neutrophils, monocytes, etc). It can only be concluded that, for immunomodulatory substances, factors other than merely maintaining some critical plasma drug concentration are important for inducing biological effects.

In terms of the mechanisms through which PGG-Glucan mediates its *in vivo* anti-infective effects, not only increased WBC numbers but also enhanced leukocyte microbicidal function appear to be involved. We observed that rats treated with PGG-Glucan exhibited higher absolute neutrophil and monocyte counts (Table 8) and enhanced neutrophil oxidative burst responses (Fig. 2) compared to rats treated with saline. It is interesting to note that increased leukocyte counts and enhanced neutrophil oxidative responses were observed in PGG-Glucan-treated rats only subsequent to bacterial challenge. These responses suggest that *in vivo* PGG-Glucan also appears to prime cells for more robust hematopoietic proliferation/differentiation as well as enhanced function when exposed to a second stimuli occurring subsequent to infection. The factor(s) inducing these responses is(are) unknown, however, augmentation of TNF-α and IL-1β proinflammatory cytokine responses does not appear to be involved (Fig. 3). These results differ significantly from those observed with other β-(1–3) glucans as well as other protein immunomodulators, such as IFN-γ, which can directly stimulate proinflammatory cytokine responses (Sherwood et al., 1987; Ijzermans & Marquet, 1989; Rasmussen et al., 1990; Rasmussen & Seljelid, 1991; Williams et al., 1996; Kilgore et al., 1997; Kulicke et al., 1997).

In conclusion, we have demonstrated that PGG-Glucan enhances leukocyte production and function and alone can protect against intra-abdominal infection caused by a multiple antibiotic

resistant *S. aureus* in rats. Furthermore, when used in combination with antibiotics, synergistic protection can be induced by PGG-Glucan. PGG-Glucan's protective effects appear to be mediated *via* enhancement of both leukocyte numbers and leukocyte function. These results suggest that PGG-Glucan may provide a new strategy for the treatment of both conventional as well as antibiotic resistant pathogens.

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