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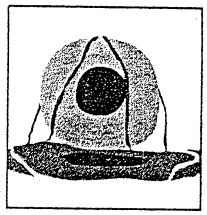

In vitro and in vivo hematopoietic activities of Betafectin® PGG-glucan

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Abstract

Betafectin® PGG-glucan is a novel β-(1,3)glucan that has broad-spectrum anti-infective activities without cytokine induction. Here we report that PGG-glucan also has both in vitro and in vivo hematopoietic activities. In vitro studies with bone marrow target cells from the C3H/HeN mouse revealed that although PGG-glucan alone had no direct effect on hematopoietic colony-forming cell (CFC) growth, when combined with granulocyte colony-stimulating factor (CSF) or granulocyte-macrophage CSF, it increased CFC numbers 1.5- to 2.0-fold over those obtained with CSFs alone. Bone marrow cells cultured for high-proliferative-potential CFCs in the presence of interleukin (IL)-1, IL-3, macrophage CSF, and stem cell factor (SCF), or cultured for erythroid burst-forming units in the presence of IL-3, SCF, and erythropoietin, also exhibited enhanced growth in the presence of PGG-glucan. The synergistic effect of PGG-glucan was specific and could be abrogated by anti–PGG-glucan antibody. The ability of PGG-glucan to modulate hematopoiesis in vivo was evaluated in myelosuppressed rodents and primates. C3H/HeN female mice were intravenously administered saline solution or PGG-glucan (0.5 mg/kg) 24 hours before the intraperitoneal administration of cyclophosphamide (200 mg/kg), and the recovery of bone marrow cellularity and granulocyte-macrophage progenitor cells was evaluated on days 4 and 8 after cyclophosphamide treatment. At both time points, enhanced hematopoietic recovery was observed in PGG-glucan–treated mice compared with saline-treated control mice. In a final series of in vivo experiments, we evaluated the ability of therapeutically administered PGG-glucan to enhance hematopoietic recovery in cyclophosphamide-treated cynomolgus monkeys. Monkeys received intravenous infusions of cyclophosphamide (55 mg/kg) on days 1 and 2, followed on days 3 and 10 by intravenous infusion of PGG-glucan (0.5, 1.0, or 2.0 mg/kg). Compared with those in saline-treated monkeys, accelerated white blood cell recovery and a reduction in the median duration of neutropenia were observed in PGG-glucan–treated monkeys. These studies illustrate that PGG-glucan has both in vitro and in vivo hematopoietic activities and that this agent may be useful in the prevention and/or treatment of chemotherapy-associated myelosuppression.

Key words: Hematopoiesis—Myelosuppression—Chemotherapy—Cyclophosphamide—β-Glucan—PGG-glucan—Betafectin®

Introduction

Yeast-derived β-(1,3)glucans are a novel class of carbohydrate compounds that have been shown to exert potent hematopoietic and anti-infective activities in animals following single-dose administration [1–11]. Particulate and derivatized (for example, aminated, sulfated, phosphorylated) β-(1,3)glucans have been shown to increase the number of bone marrow multilineage stem cells, committed progenitor cells and mature peripheral white blood cells (WBCs) in normal mice [1–4,6]. Some of these β-(1,3)glucans have also been shown to accelerate WBC, red blood cell (RBC), and platelet recovery in mice following marrow ablative radiation exposure [5,7,8]. Furthermore, a single administration of particulate or phosphorylated β-(1,3)glucan has been demonstrated to mobilize granulocyte-macrophage colony-forming cells (GM-CFCs) and spleen colony-forming units [2,4]. The hematopoietic activities of particulate and derivatized β-(1,3)glucans are thought to be related to their strong cytokine-inducing activities [3,6,10,12–15].

Betafectin® PGG-glucan (poly-[1-6]-β-D-glucopyranosyl-[1-3]-β-D-glucopyranose glucan) is a novel, nonderivatized β-(1,6) branched β-(1,3)glucan isolated from the cell walls of a proprietary strain of the yeast *Saccharomyces cerevisiae* [16,17]. It exists as a triple helical conformation, has a defined size range of 170 ± 20 kDa, and is completely soluble in aqueous solutions within the physiologic pH range. PGG-glucan has been shown to possess broad anti-infective activities without the induction of proinflammatory cytokines [16,18–25] and is currently being evaluated in a multicenter Phase III clinical trial for the ability to reduce the incidence of postsurgical infection.

Despite its lack of cytokine-inducing activity, PGG-glucan has also been shown to possess hematopoietic activities. Preliminary studies showed that in vitro, PGG-glucan could synergize with myeloid growth factors to enhance hematopoietic cell proliferation [26,27] and that in vivo, PGG-glucan could promote myelopoiesis in normal rodents [18,23]. The purpose of these studies was to further evaluate the in vitro and in vivo hematopoietic activities of PGG-Glucan. In this report, we summarize the results of studies describing the ability of PGG-glucan to synergize with multiple hematopoietic growth

factors in vitro to enhance progenitor cell growth and its ability to prophylactically or therapeutically accelerate hematopoietic recovery in mice and primates subjected to marrow-ablative chemotherapy.

Materials and methods

PGG-glucan

Soluble PGG-glucan (Betafectin®) was obtained from the manufacturer (Alpha-Beta Technology, Worcester, Massachusetts). It was received at a concentration of 1.0 mg/mL and was diluted to desired concentrations in sterile pyrogen-free saline solution (McGaw, Irvine, California). The endotoxin level in the PGG-glucan was less than 0.06 endotoxin units/mg (that is, below the limit of detection) based on a Limulus lysate endotoxin detection assay. In murine studies, PGG-glucan was intravenously administered as a single 0.5-mg/kg dose in a 0.5-mL volume. Control mice were injected intravenously with 0.5 mL of a sterile pyrogen-free saline solution. In primate studies, PGG-glucan was intravenously infused over one hour at doses of 0.5, 1.0, or 2.0 mg/kg administered in a total volume of 20 mL. Control monkeys received infusions of sterile pyrogen-free saline solution.

PGG-glucan conformers

In some in vitro studies, conformers of PGG-glucan, which were more or less structurally complex than triple-helical PGG-glucan Betafectin®, were evaluated. These included multimeric, triple-helical PGG-glucan (molecular weight >500,000) and single-helical PGG-glucan (molecular weight about 10,000). These materials were manufactured at Alpha-Beta Technology.

Anti–PGG-glucan antibody

According to the method of Lane [28], a specific anti–PGG-glucan Betafectin® monoclonal IgM antibody was produced at Alpha-Beta Technology. This antibody, BfD1, was specific to the triple-helical structure of PGG-glucan Betafectin® and did not recognize other PGG-glucan conformers. Monoclonal antispectrin IgM (Chemicon International, Temecula, California) was used as a nonspecific control antibody.

Growth factors

Growth factors used for in vitro studies included recombinant human granulocyte colony-stimulating factor (G-CSF; Amgen, Thousand Oaks, California), recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, Minnesota), recombinant human macrophage colony-stimulating factor (M-CSF; Genzyme, Cambridge, Massachusetts), recombinant human interleukin-1α (IL-1; R&D Systems), recombinant murine interleukin-3 (IL-3; R&D Systems), recombinant murine stem cell factor (SCF; R&D Systems), and recombinant human erythropoietin (EPO; R&D Systems). Growth factors were diluted in a sterile pyrogen-free saline solution (McGaw) and used at the concentrations indicated.

Chemotherapy

Myelosuppression was induced in vivo by the administration of cyclophosphamide (Cytoxan; Mead Johnson, Princeton, New Jersey). Lyophilized cyclophosphamide was diluted to a stock concentration of 20 mg/mL with sterile pyrogen-free water (Baxter Healthcare, Deerfield, Illinois) and was then further diluted to desired concentrations using sterile pyrogen-free saline solution. Mice were administered a single intraperitoneal dose of 200 mg/kg of cyclophosphamide in a 0.5-mL volume. In primates, cyclophosphamide was intravenously infused over one hour at a dose of 55 mg/kg administered in a total volume of 20 mL. Each monkey received two cyclophosphamide infusions, administered on days 1 and 2 of the study.

Mouse studies

Seven-week-old C3H/HeN female mice were purchased from Taconic Farms (Germantown, New York). Mice were barrier reared and reported by the supplier to be free of adventitious viruses and pathogenic organisms. Animals were maintained in accordance with guidelines of the National Institutes of Health (NIH) [29], the Public Health Service (PHS) [30], and the Animal Care and Use Committee of Alpha-Beta Technology. Ten animals per cage were housed in plastic Micro-Barrier cages (Allentown Caging Equipment, Allentown, New Jersey) on hardwood-chip contact bedding (Sani-Chip, PJ Murphy Forest Products, Montville, New Jersey) and were provided food (RMH 3000 Laboratory Rodent Chow, Prolab Animals Diets, Agway, Syracuse New York) and fresh tap water (city of Worcester, Massachusetts) ad libitum. Mice were allowed to acclimate to the environment of the Alpha-Beta Technology animal facility for a week before being entered into experimental protocols that were approved by the Institute Animal Care and Use Committee.

At the time of experimentation, mice were 8 weeks of age and weighed approximately 20 g. For in vitro studies, bone marrow was harvested from nontreated (normal) mice. For in vivo studies, mice were injected with PGG-glucan 24 hours before cyclophosphamide administration, and hematopoietic recovery was analyzed in three mice per treatment group on days 4 and 8 after cyclophosphamide treatment. At these times, mice were euthanized by cervical dislocation and the femurs removed and placed in Roswell Park Memorial Institute medium (RPMI; JRH Bioscience, Kansas City, Missouri) and kept on ice until bone marrow suspensions were prepared and cultured for GM-CFCs, as described below.

Primate studies

Male colony-bred cynomolgus monkeys (*Macaca fascicularis*) were purchased from Primate Products (Miami, Florida). Animals were maintained at TSI Mason Laboratories (Worcester) in accordance with NIH and PHS guidelines [29,30]. Animals were individually housed in stainless-steel cages and fed Certified Primate Chow (Purina Mills, Richmond, Indiana) with free access to fresh tap water. After arrival, monkeys were held in quarantine, during which time viral and parasitic screens were performed and each monkey was subjected to three intradermal tuberculin tests conducted at approximately two-week intervals. The monkeys were determined to be negative for measles, simian immunodeficiency virus, simian respiratory virus types 1 and 2, simian T-lymphocytic virus type 1, and free of *Campylobacter* species. Monkeys were released from quarantine after the third consecutive negative tuberculin test. Each animal was identified by a unique number and assigned into treatment groups based on random number selection.

At the time of experimentation, the mean body weight of animals used in the study was 4.1 ± 0.2 kg. Each treatment group consisted of a total of four to five animals. However, because of logistical limitations primarily related to surgical procedures, the study was performed in three stages separated by weekly intervals; saline-treated controls were included in each stage. One week before the initiation of chemotherapy (day −7), animals were surgically implanted with jugular catheters through which cyclophosphamide was infused on days 1 and 2 of the study, and PGG-glucan was infused on days 3 and 10 of the study. At the times indicated, blood specimens were obtained from each animal, and WBC, RBC, and platelet counts were obtained using an automated cell counter. A blood smear was also prepared for differential cell counting. In some instances, neutrophil oxidative burst activity was measured using a luminol-enhanced chemiluminescence assay. For this assay, blood was collected into tubes containing sodium citrate (Vacutainers, Becton Dickinson, Rutherford, New Jersey). The blood (80% vol/vol) was mixed with Hanks' balanced salt solution without Ca^{++} and Mg^{++} ($HBSS^{-}$; Sigma Chemical, St Louis, Missouri) supplemented with 10-mM HEPES (Sigma Chemical) and 75-μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical); 100-μL aliquots of the diluted blood were then plated in triplicate into wells of a 96-well Micro-

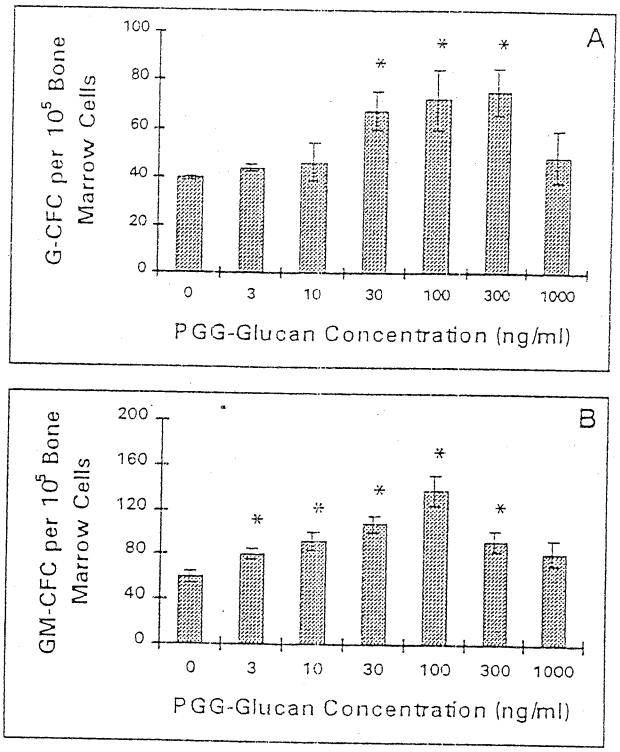

Fig. 1. Effects of PGG-glucan and G-CSF **(A)** or GM-CSF **(B)** on hematopoietic colony growth in vitro. Bone marrow cells from female C3H/HeN mice were cultured in agar in the presence of G-CSF (5 ng/mL) or GM-CSF (0.05 ng/mL), and the indicated concentrations of PGG-glucan. Colonies (>50 cells) were counted after seven days of culture. The data represent the mean ± SEM of values obtained from two replicated G-CSF experiments and from three replicated GM-CSF experiments in which each specimen was cultured in triplicate. $*p < 0.05$ with respect to cultures containing no PGG-glucan.

lite 1 plate (Dynatech Laboratories, Chantilly, Virginia). The plate was covered and placed at 37°C for 15 minutes for temperature equilibration. Cells were then stimulated with either 50 µL of $HBSS^-$ or 50 µL of opsonized zymosan (ZAP, Cardinal Associates, Santa Fe, New Mexico), and chemiluminescence readings were recorded at 30-second intervals for 20 minutes in a 37°C temperature-regulated luminometer (ML3000, Dynatech Laboratories).

In vitro hematopoietic assays

Hematopoietic colony assays were performed with bone marrow cells flushed from femurs using RPMI media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah). Each cell suspension represented a pool of cells from the femurs of three mice. The total number of nucleated cells in each suspension was determined using a Coulter counter (Coulter Electronics, Hialeah, Florida). Unless stated otherwise, bone marrow colony-forming progenitor cells (CFCs) were assayed by a modification of the semisolid agar technique [31,32]. The agar medium mixture for cell suspensions consisted of equal volumes of 0.66% agar (Difco, VWR Scientific, Philadelphia, Pennsylvania) and double-strength supplemented Connaught Medical Research Laboratory No. 1066 medium (CMRL 1066; GIBCO, Life Technologies, Baltimore, Maryland) The CMRL medium was supplemented with final concentrations of 10% heat-inactivated FBS, 5% trypticase soy broth (Sigma Chemical), 5% heat-inactivated horse serum (Hyclone), antibiotics (GIBCO), L-serine (Sigma Chemical), sodium bicarbonate (J. T. Baker, VWR Scientific), and Eagle's minimal essential medium sodium pyruvate (GIBCO). One milliliter of this media mixture containing cells was plated in triplicate into 35-mm gridded plates containing growth factors for the progenitor cells of interest.

To ensure the accuracy of colony counting, the number of cells yielding about 50 colonies per plate was determined in preliminary studies for each type of progenitor cell assay. For granulocyte progenitors (G-CFCs), 10^5 cells were plated in the presence of 5 ng/mL of G-CSF; for GM-CFCs, 10^5 cells were plated in the presence of 0.05 ng/mL of GM-CSF; for high-proliferative-potential progenitors (HPP-CFCs), 3×10^5 cells were plated in the presence of 1 ng/mL of IL-1, 1 ng/mL of IL-3, 1 ng/mL of M-CSF, and 10 ng/mL of SCF; and for erythroid burst-forming units (BFU-es), 2×10^5 cells were plated in the presence of 1.5 ng/mL of IL-3, 15 ng/mL of SCF, and 1.5 U/mL of EPO. The growth factor concentrations used were determined in preliminary studies to stimulate half maximal colony formation (SI_{50}) with the indicated target cell numbers. G-CFCs, GM-CFCs, and BFU-es were scored after seven days of incubation at 37°C in a humidified environment containing 5% CO_2; HPP-CFCs were scored after 14 days of incubation. For comparison purposes, all data are reported as colony numbers per 10^5 cells.

Data analysis

Results are presented as the mean ± SEM of data obtained in replicate experiments. Statistical analyses were performed by Student's two-tailed *t* test or analysis of variance using commercially available software (Instat, GraphPad, San Diego, California).

Results

In vitro effects of PGG-glucan

Initial studies evaluated the ability of PGG-glucan to directly stimulate in vitro colony formation by murine bone marrow cells. These studies revealed that PGG-glucan alone had no in vitro hematopoietic activity (data not shown). Subsequent studies investigated the ability of PGG-glucan to enhance colony growth induced in vitro by single myeloid growth factors. In the presence of either G-CSF or GM-CSF, PGG-glucan dose-dependently increased murine bone marrow G-CFC and GM-CFC colony formation (Fig. 1A and 1B). PGG-glucan at the concentration of 100 ng/mL increased colony formation 1.5- to 2.0-fold compared with the CSFs alone. Additional studies evaluated the ability of PGG-glucan to enhance colony formation by immature myeloid and erythroid progenitors requiring rich cytokine mixtures for growth. In these studies, both HPP-CFC and BFU-e colony formations were also shown to be increased 1.5- to 2.0-fold in the presence of PGG-glucan (Fig. 2A and 2B). Interestingly, in the presence of optimal growth factor concentrations (that is, SI_{100} concentrations), PGG-glucan failed to enhance colony formation beyond that observed with the growth factors alone. These studies illustrated that, at limiting growth factor concentrations, PGG-glucan was capable of enhancing the responsiveness of immature and mature myeloid as well as immature erythroid progenitor cells to growth factor–induced proliferation.

The in vitro hematopoietic activity of PGG-glucan was further investigated using the GM-CFC assay system. Additional experiments demonstrated that PGG-glucan not only increased the number of progenitors forming colonies in the presence of GM-CSF (as illustrated in Fig. 1B), but the number

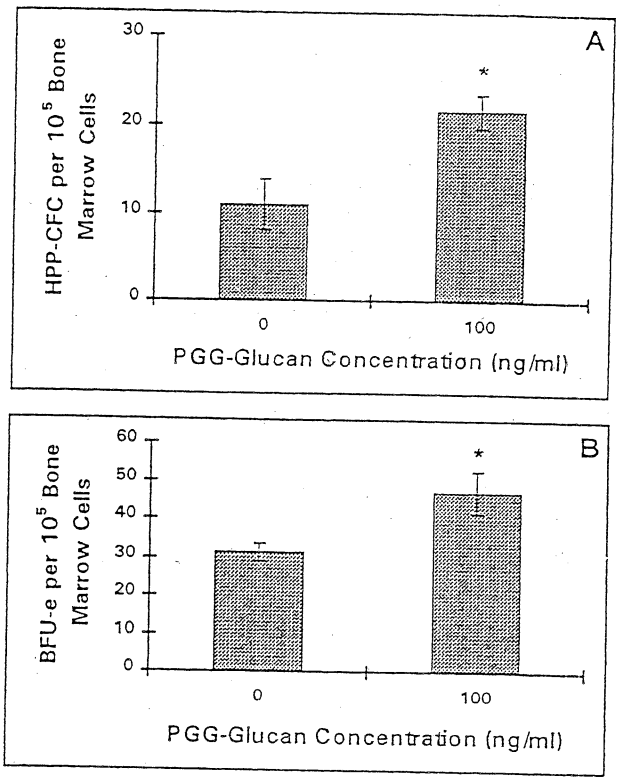

Fig. 2. Effects of PGG-glucan on HPP-CFC **(A)** and BFU-e **(B)** growth in vitro. Bone marrow cells from female C3H/HeN mice were cultured in agar in the presence of 100 ng/mL of PGG-glucan and 1 ng/mL of IL-1, 1 ng/mL of IL-3, 1 ng/mL of M-CSF, and 10 ng/mL of SCF to grow HPP-CFC and in the presence of 100 ng/mL of PGG-glucan and 1.5 ng/mL of IL-3, 15 ng/mL of SCF, and 1.5 U/mL of EPO to grow BFU-e. HPP-CFCs (>50,000 cells) were scored after 14 days of culture; BFU-es were scored after 7 days of culture. The data represent the mean ± SEM of values obtained from two replicated experiments in which each specimen was cultured in triplicate. $*p < 0.05$ with respect to cultures containing no PGG-glucan.

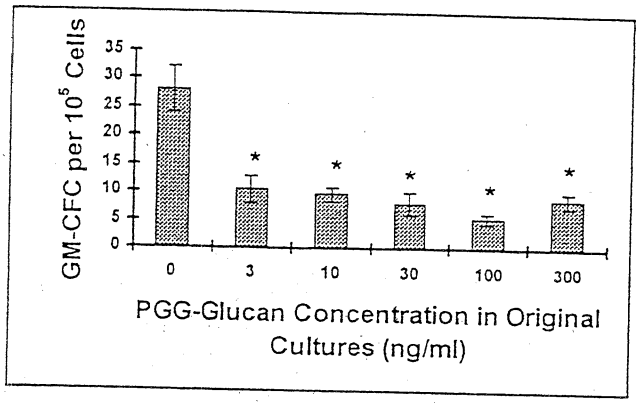

Fig. 3. Effects of PGG-glucan on the replating potential of GM-CFCs. Bone marrow cells from female C3H/HeN mice were cultured in methylcellulose media (Stem Cell Technologies, Vancouver, Canada) in the presence of GM-CSF (0.05 ng/mL) and PGG-glucan (100 ng/mL). After 7 days of culture, cells were washed from the methylcellulose, resuspended, and plated in the agar GM-CFC assay in the presence of GM-CSF (0.05 ng/mL). In both assays, cells were cultured at a density of 10^5/mL. GM-CFC (>50 cells) were counted after 7 days of culture. The data represent the mean ± SEM of values obtained from two replicated experiments in which each specimen was cultured in triplicate. $*p < 0.05$ with respect to cultures containing no PGG-glucan.

of cells per colony was also increased about 1.5-fold in the presence of PGG-glucan. The average number of cells per colony in cultures containing GM-CSF alone was 114 ± 7 compared with 170 ± 12 in cultures containing both GM-CSF and 100 ng/mL of PGG-glucan ($p < 0.05$). Furthermore, the ability of PGG-glucan to enhance the differentiation of myeloid progenitors was suggested by the results of replating experiments (Fig. 3). In these experiments, compared with cells cultured for seven days in the presence of GM-CSF alone, fewer cells cultured for seven days in the presence of GM-CSF and PGG-Glucan retained colony-forming capacity when recultured. Whether this reflected only effects on cells within the primary colonies or possibly also effects on cells within the intracolony spaces could not be determined from these studies.

The specificity of PGG-glucan's effect was demonstrated by ability of BfD1, a specific anti–PGG-glucan Betafectin® antibody, to completely abrogate the hematopoietic synergy induced by PGG-glucan (Fig. 4A). Additional studies demonstrated that the hematopoietic synergy induced by PGG-glucan was specific to the triple-helical conformation of PGG-glucan Betafectin® because no synergistic hematopoietic effects were obtained with the single-helical β-(1,3)PGG-glucan conformer or with the multimeric triple-helical β-(1,3)PGG-glucan conformer (Fig. 4B).

In vivo effects of PGG-glucan

In vivo effects of PGG-glucan were initially evaluated in cyclophosphamide-myelosuppressed mice. PGG-glucan was prophylactically administered to mice 24 hours before chemotherapy treatment, and the recovery of bone marrow cellularity and GM-CFC levels was monitored. A 0.5-mg/kg PGG-glucan dose was chosen for these studies based on hematopoietic changes previously observed in normal rodents administered this PGG-glucan dose [18,23]. By day 4 after chemotherapy, marrow cellularity in saline-treated mice had recovered to about 35% of normal control values whereas marrow cellularity in mice treated with PGG-glucan had recovered to about 50% of normal control values (Table 1). By day 8, recovery to normal bone marrow cellularity was observed in saline-treated mice, and greater than normal cellularity was observed in PGG-glucan–treated mice. Although greater than normal GM-CFC numbers were evident in the marrow of all mice at the day 4 evaluation point, GM-CFC levels in PGG-glucan–treated mice (about 260% of normal) were significantly higher than in saline-treated control mice (about 175% of normal; Table 1). By day 8, when GM-CFC proliferation had subsided in all mice, GM-CFC progenitor numbers remained significantly elevated in PGG-glucan–treated mice compared with saline-treated mice.

Further studies were performed to investigate the ability of PGG-glucan to therapeutically enhance hematopoietic recov-

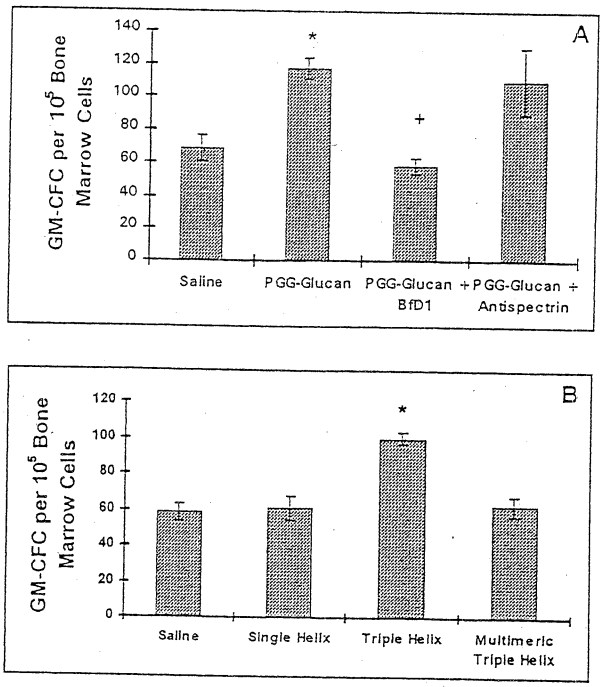

Fig. 4. PGG-glucan specificity studies. **A.** Effects of anti–PGG-glucan antibody on PGG-glucan–enhanced GM-CFC colony formation. Bone marrow cells from female C3H/HeN mice were cultured in agar in the presence of GM-CSF (0.05 ng/mL) and PGG-glucan (100 ng/mL) alone or in the presence of anti–PGG-glucan IgM antibody (BfD1; 1:2500 dilution); control cultures contained anti-spectrin IgM antibody (1:2500 dilution). GM-CFCs (>50 cells) were counted after seven days of culture. The data represent the mean ± SEM of values obtained from two replicated experiments in which each specimen was cultured in triplicate. $*p < 0.05$ with respect to control cultures, $^{+}p < 0.05$ with respect to cultures containing only PGG-glucan or PGG-glucan plus antispectrin antibody. **B.** The effects of various PGG-glucan conformers on GM-CFC colony formation. Bone marrow cells from female C3H/HeN mice were cultured in agar in the presence of GM-CSF (0.05 ng/mL) alone or together with the indicated PGG-glucan conformers (100 ng/mL). GM-CFC (>50 cells) were counted after seven days of culture. The data represent the mean ± SEM of values obtained from two replicated experiments in which each specimen was cultured in triplicate. $*p < 0.05$ with respect to cultures containing only recombinant murine GM-CSF.

ery following myeloablative chemotherapy. In these studies, a more clinically relevant model of prolonged myelosuppression was induced in cynomolgus monkeys. Compared with saline-treated monkeys, monkeys treated with PGG-glucan exhibited an accelerated WBC recovery, which was most pronounced in animals treated with the 0.5-mg/kg and the 1-mg/kg PGG-glucan doses (Fig. 5A). Based on differential analysis, it appeared that this effect was primarily due to an acceleration of neutrophil recovery (Fig. 5B). Although not implicitly obvious from the mean values presented in Figure 5, PGG-glucan treatment also reduced the duration of neutropenia as measured by the median number of days animals within each treatment group exhibited absolute neutrophil counts (ANCs) of less than 500/μL. The median duration of neutropenia was reduced from 6 days in saline-treated monkeys to 2.5, 1.5, and 1.5 days, respectively, in monkeys treated with 0.5, 1.0, and 2.0 mg/kg of PGG-glucan. Based on WBC recovery, ANC recovery, and the duration of neutropenia, the 1-mg/kg PGG-glucan dose appeared to be most effective. The recovery of the neutrophil oxidative function also appeared to be accelerated in animals receiving the 1-mg/kg PGG-glucan treatment (Fig. 6). RBC and platelet recoveries were not altered by any PGG-glucan treatment (data not shown).

Discussion

PGG-glucan Betafectin® is a highly purified soluble β-(1,6) branched, β-(1,3)glucan polymer isolated from *S cerevisiae*. This immunomodulator has been shown to have broad in vitro and in vivo anti-infective activities without inducing proinflammatory mediators [16,18,19, 22–25]. In Phase II clinical trials, PGG-glucan has been shown to reduce postoperative infection rates and to shorten the length of hospital stays [20,21]; Phase III clinical trials are currently in progress. PGG-glucan interacts with leukocytes through receptors that appear to be distinct from the receptors for other β-(1,3)glucans [33,34] and is known to activate NF-κB and NF–IL-6 nuclear transcription factors [35]. Although PGG-glucan does not directly stimulate neutrophil function, it has been shown to prime neutrophils for activation by secondary stimuli [16,22,24], a characteristic that distinguishes PGG-glucan from other β-(1,3)glucans, which directly activate cellular functions [9–15]. Other β-(1,3)glucans have also been shown to possess potent hematopoietic activities [1–8]. These hematopoietic activities have been presumed to be related to their strong cytokine-inducing abilities [3 6,10,12–15,36]. In this report, we present evidence that PGG-glucan, a noncytokine-inducing β-(1,3)glucan, also possesses hematopoietic activity in vitro and in vivo.

Although PGG-glucan alone did not exhibit hematopoietic activity in vitro, it enhanced progenitor proliferation 1.5- to 2.0-fold in the presence of either single or multiple growth factors, stimulating G-CFC, GM-CFC, HPP-CFC, and BFU-e proliferation. These studies illustrated that PGG-glucan is capable of functioning as a synergistic factor to enhance the in vitro proliferation of immature and mature myeloid as well as immature erythroid progenitor cells. PGG-glucan appeared to increase the number of progenitors responding to growth factors and to increase the proliferative potential of individual progenitors. Such synergistic hematopoietic activity has previously been documented for the protein growth factors c-kit ligand (that is, steel factor, mast cell growth factor, SCF) and flt-3 ligand [37–40]. These protein factors are proposed to enhance colony formation by increasing progenitor cell survival and by altering progenitor cell cycle status. Whether the synergistic effects of PGG-glucan may be mediated by similar mechanisms is not known at this time.

In addition to in vitro hematopoietic effects, PGG-glucan induced in vivo hematopoietic activity. The murine studies presented in this report illustrated that prophylactic PGG-glucan treatment can enhance myeloid recovery following myelosuppressive cyclophosphamide treatment. Compared with saline-treated control mice, mice receiving PGG-glucan exhibited increased bone marrow cellularity and GM-CFC

Table 1. Effect of PGG-glucan treatment on bone marrow cellularity and GM-CFC recovery in cyclophosphamide-treated mice*

	Normal control†	Saline	PGG-glucan
Femur cellularity, $\times 10^6$			
Day 4	14.76 ± 0.80	5.00 ± 0.57	6.85 ± 1.00
Day 8	NE‡	14.72 ± 0.32	16.24 ± 0.22§
GM-CFC/femur			
Day 4	8,360 ± 827	14,615 ± 1,249	21,537 ± 2,166§
Day 8	NE‡	3,863 ± 72	5,345 ± 428§

*C3H/HeN female mice were injected intravenously with 0.5 mg/kg of PGG-glucan 24 hours before the intraperitoneal administration of 200 mg/kg of cyclophosphamide. The data represent the mean ± SEM of values obtained from three experiments.
†Values from normal mice (that is, mice receiving no drug treatments).
‡NE = not evaluated.
§$p < 0.05$, with respect to saline.

levels after chemotherapy treatment. An ability to enhance hematopoietic regeneration when administered before exposure to myelosuppressive agents has been observed with numerous immunomodulatory agents, including endotoxin, killed microbes, microbial extracts, and particulate or derivatized β-(1,3)glucans [36]. Like these agents, PGG-glucan also mobilizes progenitor cells into the peripheral circulation (Patchen et al., unpublished data). However,

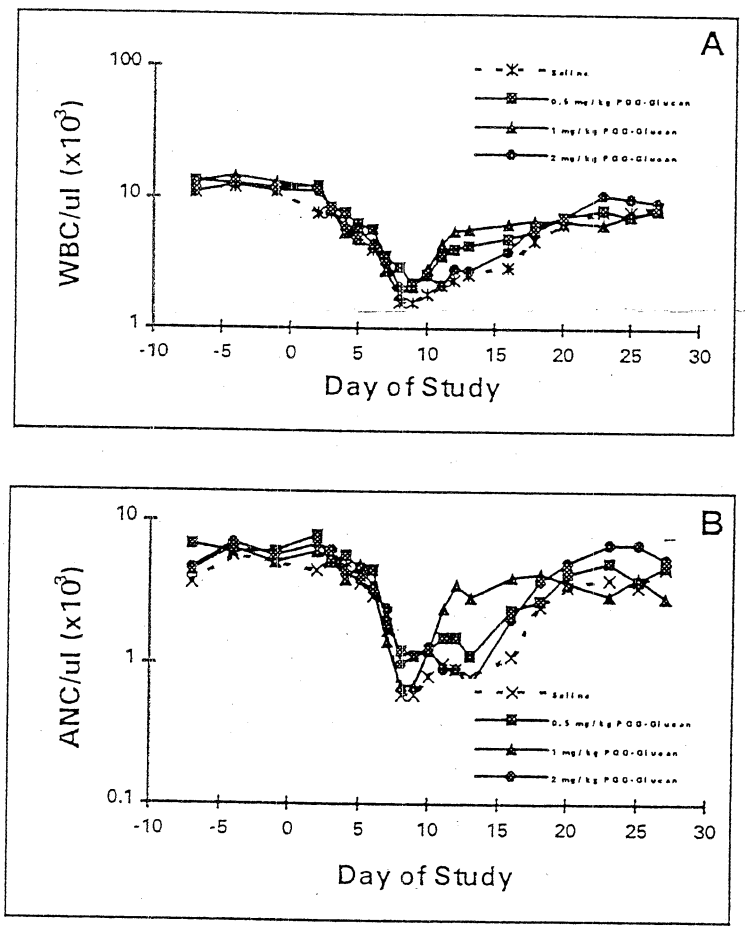

Fig. 5. Effect of PGG-glucan treatment on WBC recovery (A) and ANC recovery (B) in cyclophosphamide-treated cynomolgus monkeys. Monkeys received intravenous infusions of cyclophosphamide at a dose of 55 mg/kg on study days 1 and 2 and intravenous infusions of PGG-glucan (0.5, 1, or 2 mg/kg) on study days 3 and 10. At the times indicated, blood was drawn from each animal to perform complete and differential blood cell counts. The data represent the mean of values obtained from five saline-treated and four PGG-glucan–treated monkeys per group.

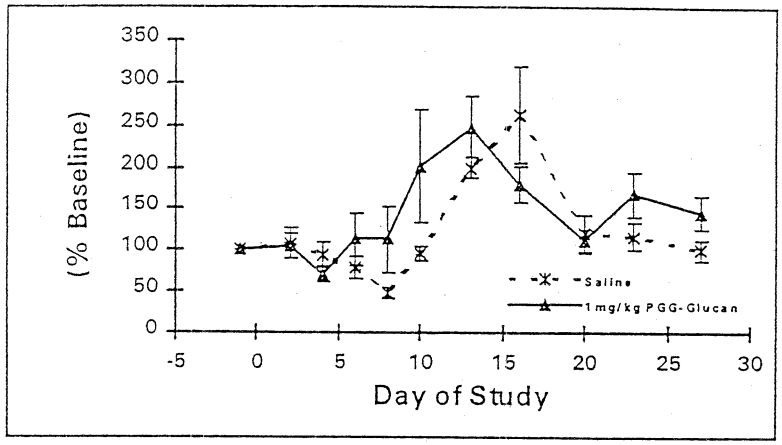

Fig. 6. Effect of PGG-glucan treatment on neutrophil oxidative burst responses in cyclophosphamide-treated cynomolgus monkeys. Monkeys received intravenous infusions of cyclophosphamide at a dose of 55 mg/kg on study days 1 and 2 and intravenous infusions of PGG-glucan (1 mg/kg) on study days 3 and 10. At the times indicated, blood was drawn from each animal to assay neutrophil oxidative burst activity, as described in the "Materials and methods" section. On each assay day, the response from each animal was normalized for the number of neutrophils assayed and the response calculated as a percentage of the baseline response observed before chemotherapy treatment. The data represent the mean ± SEM of values obtained from five saline-treated and four PGG-glucan–treated monkeys. The p on days 8, 10, 23, and 27 was 0.05, 0.06, 0.05, and 0.04, respectively.

whether progenitor cell mobilization per se contributes to the hematopoietic recovery observed with the prophylactic administration of these agents is not known.

In addition to its prophylactic effects, PGG-glucan was shown in the primate studies reported here to have the ability to therapeutically enhance hematopoietic recovery following myelosuppressive chemotherapy. Compared with saline-treated control monkeys, monkeys that were not initiated on PGG-glucan treatment until the day after the cessation of chemotherapy also exhibited enhanced hematopoietic activity, as evidenced by an increase in WBC recovery, ANC recovery, and a reduction in the median duration of neutropenia. The recovery of neutrophil function was also accelerated in PGG-glucan–treated monkeys compared with saline-treated controls. The regenerative effects observed with PGG-glucan treatment occurred following the administration of only two doses of PGG-glucan. Thus, PGG-glucan differs from classical growth factors (for example, G-CSF, GM-CSF) that typically require multiple daily administrations to induce hematopoietic regeneration and whose effects quickly subside after the cessation of administration. Whether the effectiveness of PGG-glucan could be further improved by multiple injections remains to be determined.

Although it may at first seem contradictory that PGG-glucan alone could induce in vivo effects but could not induce in vitro effects, its in vivo effects may actually result from its ability to enhance progenitor cell responsiveness to endogenous cytokines. This seems especially possible given that endogenous cytokine production has been shown to be significantly up-regulated following marrow ablative injury [41–44]. This concept is further substantiated by a previous study in which additive synergistic hematopoietic responses have been observed in mice administered β-glucan in combination with the growth factor G-CSF [45].

Taken together, these results demonstrate that PGG-glucan can enhance hematopoiesis both in vitro and in vivo. Combined with the anti-infective activity of PGG-glucan, these results suggest that the prophylactic and/or therapeutic application of PGG-glucan for the treatment of chemotherapy-associated myelosuppression and subsequent susceptibility to opportunistic infections may be useful.

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