MARINE BIOTECHNOLOGY

Novel Sulfated Polysaccharide Derived from Red-Tide Microalga *Gyrodinium impudicum* strain KG03 with Immunostimulating Activity in vivo

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Received: 14 May 2004 / Accepted: 27 September 2004 / Online publication: 4 June 2005

Abstract

The high-sulfate-containing exopolysaccharide p-KG03 is produced by the red-tide microalga *Gyrodinium impudicum* strain KG03. The immunostimulatory effects of this sulfated exopolysaccharide were investigated by isolating peritoneal macrophages from mice 10 or 20 days after they had received a single dose of p-KG03 (100 or 200 mg/kg body weight). The cytotoxicity of the isolated macrophages for B16 tumor cells was tested, as B16 tumor cells are sensitive to tumor necrosis factor α (TNF-α) and nitric oxide. The activities of natural killer cells from the p-KG03-treated mice against YAC-1 mouse lymphoma cells were also tested. The nonspecific immune functions mediated by natural killer cells and macrophages were increased by treatment with p-KG03 in vivo. These results suggest that p-KG03 has immunostimulatory effects and enhances the tumoricidal activities of macrophages and NK cells in vivo. In addition, p-KG03 treatment increased the plaque-forming cell response to sheep red blood cells, as well as the levels of IgM and IgG Exposure to p-KG03 also increased the production by macrophages of cytokines, such as interleukins -1β and -6, and TNF-α. This is the first report of a marine microalgal sulfated polysaccharide having immunostimulatory activities. The p-KG03 polysaccharide may be useful for the development of biotechnological and pharmaceutical products that incorporate bioactive marine exopolysaccharides.

Key words: immunostimulating activity — sulfated exopolysaccharide — *Gyrodinium impudicum* — IL-1β — IL-6 — TNF-α — macrophage — natural killer cell — antitumor activity

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Introduction

Interest in microorganisms as producers of high molecular weight polysaccharides has increased in recent years because these biopolymers often show advantages over the polysaccharides that are currently in use (Sutherland, 1996). Marine algae are a source of natural products with pharmacologic applications, and include substances with immunostimulatory activities. Macrophages are the first line of host defenses against bacterial infection and tumor growth, and thus they play an important role in the initiation of adaptive immune responses (Verstovsek et al., 1992; Fidler, 1995).

Macrophages that are stimulated with bacterial products, such as lipopolysaccharide (LPS) and muramyl dipeptide, release several proinflammatory cytokines, including interleukins (IL-I, IL-6, and IL-8), tumor necrosis factor α (TNF-α), and nitric oxide (NO), which directly induce the tumoricidal activity of macrophages (Stuehr and Marletta, 1985). When activated, macrophages increase their phagocytic activities and mediate nonspecific immune responses (Adams and Hamilton, 1984). In particular, there has been great interest in the release of the reactive nitrogen intermediate NO because of its antibacterial and antitumor effects (Bredt and Snyder, 1994). Therefore, the ability of certain factors to induce cytokine production and release by macrophages is thought to reflect their roles as immunomodulators. In addition to being involved in the activation of macrophages, these cytokines are related to secondary immune responses, such as the proliferation and differentiation of T and B cells (Seljelid et al., 1989). *Phellinus linteus* is a medicinal mushroom that belongs to the *Hymenochaetaceae basidiomycetes*. Polysaccharides purified from this mushroom are capable of potentiating host immune response without direct cytotoxicity for cancer cells

(Franz, 1989). Moreover, polysaccharides stimulate the immune functions of T lymphocytes and the nonspecific immune functions that are mediated by natural killer (NK) cells and macrophages (Song et al., 1995). Alginates containing high mannuronic acid are composed of 1,4-linked β-D-mannuronic acid (M), α-L-guluronic acid (G), and alternating (MG) blocks; it has been shown that the M and MG blocks stimulate monocytes to produce IL-1β, IL-6, and TNF-α, whereas the G blocks do not have this activity (Otterle et al., 1991). It has also been reported that β-1,4-linked D-mannuronic acid and β-1,3-glucan, which are mannose-containing polysaccharides, increase the antitumor activities and cytokine production of macrophages and monocytes (Seljelid et al., 1989). In addition, mannoglucan shows antitumor and TNF-α-like activities against tumor tissues (Takahashi et al., 1988).

Sulfated polysaccharides have a wide range of important biological properties. Marine algae are rich sources of sulfated polysaccharides with novel structures, and these compounds have anticoagulant properties (McLellan and Jurd, 1992). Sulfated polysaccharides from marine macroalgae also are known to interfere with the adsorption and penetration of some enveloped viruses (Batinic and Robey, 1992). Sulfated residues are found in eukaryotic algal exopolysaccharides (Bourgougnon et al., 1993) and in some cyanobacterial polysaccharides (Sudo et al., 1995). *G. impudicum* is known to produce mucous exopolysaccharide (Park and Park, 1999), and p-KG03 is a selective inhibitor of viral replication, especially that of encephalomyocarditis virus (EMCV), in vitro (Yim et al., 2004). In this study the in vivo immunostimulatory activity of the p-KG03 exopolysaccharide from the marine microalga *G. impudicum* strain KG03 was studied, and the sulfated polysaccharide was characterized with a view to developing immunomodulatory therapies.

Materials and Methods

Algal Strain and Production of p-KG03. The microalgal strain KG03 was originally isolated from a red tide in Korea, and identified as *Gyrodinium impudicum* (Figure 1) (Yim, 2002). Briefly, bacteria-free cultures of the *G. impudium* KG03 strain were grown in M-KG03 medium in a 2-L photoreactor. The production conditions were 1% CO_2, 50 ml/min^{-1} airflow, 22.5°C, and light intensity of about 150 $\mu E/m^2/s$, with a 16-hour light/8-hour dark illumination cycle (Yim et al., 2003). The cells were removed from the culture medium by centrifugation at 12,000*g* for 30 minutes 4°C. The sulfated polysaccharide (p-KG03) was isolated from the supernatant by the addition of 2 volumes of ethanol, and precipitation at 4°C for 24 hours. The precipitated polysaccharide was collected by filtration (Whatman GFF filter), dissolved in deionized water, and reprecipitated by the addition of a 3% solution of cetyltrimethylammonium bromide (Cetavolon; Merk). The precipitated Cetavolon-polysaccharide complex was collected by centrifugation at 10,000*g* for 20 minutes at 4°C, and redissolved in 10% NaCl. The precipitated polysaccharide component was recovered by the addition of 3 volumes of ethanol. The extracted polysaccharide was dissolved in deionized water and dialyzed against deionized water for 2 days. Further fractionation and purification were achieved by gel chromatography on a Sepharose 4B column (Sigma Chemical Co), followed by elution with 0.4 M NaCl buffer. The eluate was analyzed by the phenol–sulfuric acid method (Dubios et al., 1956). The carbohydrate-containing fractions were collected, dialyzed using Viva-flow (Sartorius), and freeze-dried (Figure 1, C).

Mice and Chemicals. Male C57BL/6 mice (6–8 weeks old, 17–21 g) were obtained from Charles River Breeding Laboratories (Japan). Five animals were randomly distributed into each group. During the experiment, the animals were maintained at 23° ± 1°C, 55% ± 5% humidity, 10 to 18 air circulations per hour, and 12-hour light/dark cycles. The animals had free access to food and water. The sulfated exopolysaccharide was prepared as suspensions of 4 and 8 mg/ml in D-PBS. The animals (5 mice per group) were gavaged with a single dose of the suspension (100 or 200 mg/kg body weight) daily for 10 or 20 days. The control mice received D-PBS only.

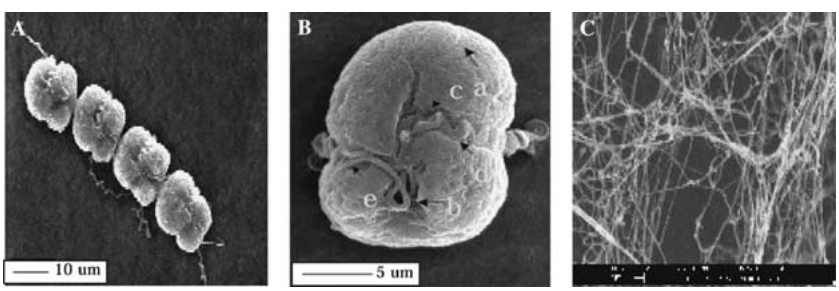

Fig. 1. Scanning electron micrographs of *Gyrodinium impudicum* strain KG03. **A:** Four-chained cells. **B:** A single cell (a, apicon; b, hypocon; c, groove; d, transverse flagellum; e, longitudinal flagellum). **C:** p-KG03 at 0.1% (w/v).

The animals were sacrificed by ether anesthesia after being exposed to p-KG03 for 10 or 20 days. The mice were observed daily for clinical signs. None of the mice showed abnormal growth, abnormal behavior, illness, or mortality during the study period. Unless otherwise indicated, all of the chemicals were purchased from Sigma. The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco. Thioglycollate broth was purchased from Difco Laboratories. All of the tissue culture reagents and the purified p-KG03 were assayed for endotoxin contamination by the *Limulus* amebocyte lysate assay (E-Toxate; Sigma).

Collection and Counting of Peritoneal Macrophages. Pooled peritoneal exudate cells were harvested from p-KG03-treated and PBS-treated mice (n = 5 for each experiment). Following lavage of the peritoneal cavity with 6 ml of RPMI 1640 medium, the cells were washed twice and resuspended in RPMI 1640 that contained 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (RPMI-FBS) (Pyo et al., 1991). Total cell counts were determined using a hemocytometer. In addition, 100 μl of the cell suspension was centrifuged onto a microscope slide for 10 minutes at 1000*g* using a Cytospin 3 centrifuge (Shandon). The cytospun cells were fixed and stained using the ACUSTAIN Wright-Giemsa Stain (Sigma) to allow calculation of the percentage of macrophages. Total numbers of peritoneal macrophages were obtained by multiplying these percentages by the total numbers of peritoneal exudate cells. Based on differential staining, the macrophages were seeded at densities of 1×10^6 cells per well into a 96-well plate (Falcon Plastics) and allowed to adhere for 2 hours at 37°C and 5% CO_2 in a humidified incubator. After 2 hours of incubation, nonadherent cells were removed by washing 3 times with culture medium. More than 98% of the adherent cell populations were identified as macrophages on the basis of morphology and phagocytic criteria (Saiki and Fidler, 1984).

Plaque Assay and Antibody Production. The ability to produce specific antibody was determined by quantifying the plaque-forming cell (PFC) response to sheep red blood cells (SRBCs) (Institoris et al., 1995). The animals were immunized with 2×10^9 SRBCs (i.p.) in 0.2 ml PBS at 10 or 20 days after exposure to p-KG03. On day 4 after immunization, the spleens were removed from the mice. Splenic cell suspensions (2×10^7 cells/ml) were prepared in sterile culture medium. An aliquot of each sample (50 μl, 10^6 cells) was mixed with 20 μl of guinea pig complement, 30 μl of 30% SRBCs, and 0.4 ml of prewarmed agar (47°C) in a 35 × 10-mm culture dish (Fisher Scientific), then incubated at 37°C with 5% CO_2 for 4 hours. After incubation the plaques were counted under a light microscope, and the number of PFCs per 10^6 cells was calculated. In addition, the supernatants from spleen cell cultures that were stimulated with LPS (10 μg/ml) for 3 days were used to determine the levels of secreted IgG and IgM by enzyme-linked immunosorbent assay (ELISA).

Assessment of NK Cell Cytotoxicity. The spleen cells of male C57BL/6 mice were used as effector cells, and YAC-1 mouse lymphoma cells (ATCC) that were labeled with sodium ^{51}Cr-chromate (Amersham Biosciences) were used as target cells. The NK cell cytotoxicity assays were performed by co-incubation of 2.5×10^5 or 5×10^5 effector cells with 1×10^4 YAC-1 target cells in a final well volume of 200 μl in 96-well round-bottom plates (Costar Products) for 6 hours at 37°C and 5% CO_2 in a humidified incubator. Effector–target cell ratios (E/T) of 25:1 and 50:1 were chosen in this study after preliminary assays. The specific release of ^{51}Cr by the YAC-1 target cells reflected the natural cytotoxic activity of the NK cells, and was calculated as

$$\%Cytoxicity = [(E - S)/(T - S)] \times 100$$

where E is the activity of ^{51}Cr released from the target cells in the presence of effector cells, S is the activity of ^{51}Cr released spontaneously under identical conditions from target cells alone, and T is the maximum activity of ^{51}Cr released when all the target cells are disrupted.

Macrophage-Mediated Cytotoxicity. The assay for macrophage cytotoxicity was performed via the modification of a previously described technique (Flick and Gifford, 1984; Klostergaard, 1985). Briefly, macrophages (1×10^5 cells per well) were plated into 96-well microtiter plates (Costar Products) for 2 hours at 37°C in a 5% CO_2 incubator. After 2 washes with warm medium, the macrophages were co-incubated with B16 mouse melanoma cells (ATCC), syngeneic to the C57BL/6 cells (1×10^4 cells per well, which is an initial effector–target cell ratio of 10:1), at 37°C in a 5% CO_2 incubator. After 20 hours of incubation, the plates were stained for 15 minutes with crystal violet that contained 10% formaldehyde. The absorbance of each well was determined at 540 nm using a microplate reader (Molecular Devices). Cytolytic activity was expressed as a percentage of tumor cytotoxicity, based on the following formula:

$$\{1 - [OD\ of\ (Target\ Cells + Macrophages) - OD\ of\ Macrophages]/OD\ of\ Target\ cells\} \times 100$$

where the OD of the target cells is the optical density of the B16 melanoma cells, and the OD of the macrophages is the optical density of the macrophages.

Cytokine Determinations by ELISA. Peritoneal macrophages from treated or untreated male B57BL/6 mice were cultured for 20 hours. The culture supernatants were collected, and the concentrations of IL-1β, IL-6, and TNF-α in the culture supernatants were determined using cytokine-specific ELISA kits according to the manufacturer's instructions.

NO Determination. The macrophages were cultured for 20 hours, and the accumulation of nitrite in the culture supernatant was measured using the assay system described previously (Ding et al., 1988). Briefly, 100 μl of supernatant was removed from each well into an empty 96-well plate. After the addition of 100 μl of Griess reagent to each well, the absorbance at 540 nm was measured using a microplate reader (Molecular Devices). The concentration of nitrite was calculated from a standard curve for $NaNO_2$; the absorbance levels were indicative of NO production. The Griess reagent was mixed was 0.1% naphthylethylenediamine dihydrochloride in distilled water and 1% sulfanilamide in 5% H_3PO_4 (1:1).

Assay for Hydrogen peroxide. The macrophages were cultured for 20 hours, and the secretion of hydrogen peroxide was measured fluorimetrically by the horseradish-peroxidase-catalyzed oxidation of fluorescent scopoletin to a nonfluorescent product, as described previously (Nathan and Root, 1977). The emission of light at 460 nm from reduced scopoletin, following excitation at 350 nm, is extinguished when scopoletin is oxidized by H_2O_2 in the presence of horseradish peroxidase. Under the assay conditions used, the loss of fluorescence was proportional to the concentrationH_2O_2.

Statistical Analyses. The results of 2 experiments performed in quintuplicate are shown with the values expressed as the means ± SE. Statistical differences between the groups were determined by the Student's t test ($p < 0.05$).

Results

Effects on Antibody Production. Since the splenic PFC response against SRBC is one of the immune functions with sensitivity to immunomodulators,

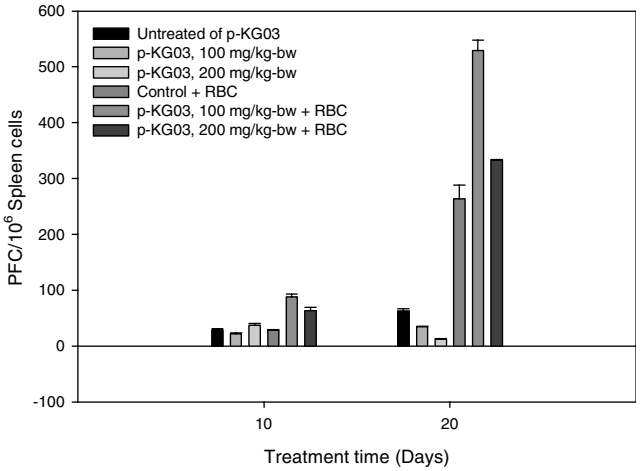

Fig. 2. PFCs per 10^6 spleen cells in mice orally administered p-KG03 at the indicated dosages for 10 or 20 days. The values shown are means ± SE for 5 mice. The experiments were repeated twice.

the effects of p-KG03 on the PFC response were examined. A significant increase was seen in the number of PFCs per 10^6 spleen cells in mice that had been exposed to p-KG03 for 20 days, when compared with untreated mice. The effect of p-KG03 on macrophage infiltration into the peritoneal cavity was assessed by counting the number of peritoneal macrophages 10 and 20 days after a single injection of p-KG03. After treatment with SRBC, the number of macrophages in the 10-day treatment groups (100 and 200 mg/kg body weight) increased 3.07-fold and 2.23-fold, respectively, and the number of macrophages in the 20-day treatment groups (100 and 200 mg/kg body weight) increased 2.01-fold and 1.26-fold, respectively, as compared with the untreated group (Figure 2). These data indicate that p-KG03 enhances macrophage function when injected into mice. In addition, the production of IgM by the 20-day treatment groups (100 and 200 mg/kg body weight) was increased about 1.13-fold and 1.22-fold, respectively, and the level of IgG was increased about 1.33-fold and 1.56-fold, respectively. Consequently, the levels of IgG and IgM in the 10-day treatment groups were not increased (Figure 3).

Effect of p-KG03 on Tumoricidal Activities of NK Cells and Macrophages. To examine the effects of p-KG03 on NK cell activity, spleen cells and YAC-1 mouse lymphoma cells were administered with 2 p-KG03 concentrations (100 and 200 mg/kg body weight) for 2 different periods of treatment (10 and 20 days). After treatment for 20 days with 100 mg/kg body weight p-KG03, NK cell cytotoxicity was increased 5.3-fold, compared with the untreated group. However, the 10-day treatment groups did not show

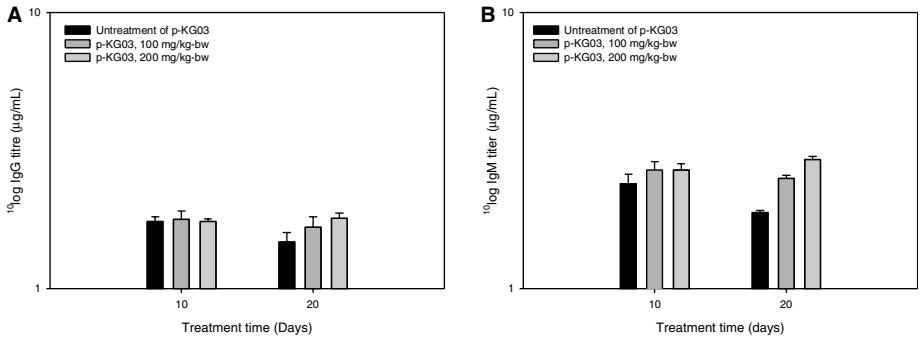

Fig. 3. IgG (**A**) and IgM (**B**) production in mice that were orally administered p-KG03 at the indicated dosages for 10 or 20 days. The values shown are means ± SE for 5 mice. The experiments were repeated twice.

increased NK cell cytotoxicity (Figure 4, A). The influence of p-KG03 on macrophage activity against B16 mouse melanoma cells was calculated as a cytotoxicity index relative to the untreated group. The cytotoxicity indices of the macrophages in the mice after 10 days of treatment with 100 and 200 mg/kg body weight p-KG03 increased 2.51-fold and 6.91-fold, respectively, whereas there was no significant increase in the cytotoxicity index after 20 days of treatment with either dosage of p-KG03 (Figure 4, B).

Effect of p-KG03 on Cytokine Production of Activated Macrophages. IL-1β production by macrophages that were cultured for 20 hours following stimulation with 100 and 200 mg/kg body weight p-KG03 for 10 days increased 35.9-fold (1110 pg/ml) and 29.4-fold (912 pg/ml), respectively, in comparison with the control (Figure 5, A). IL-6 production increased 1.45-fold in the group treated with 100 mg/kg body weight p-KG03 for 10 days, but did not increase in the other groups (Figure 5, B). TNF-α production increased 5.13-fold (524 pg/ml) and 16.3-fold (1665 pg/ml) in the groups that were treated for 20 days with 100 and 200 mg/kg body weight, respectively (Figure 5, C).

Effects of p-KG03 on NO and H_2O_2 Production. The effect of p-KG03 on the production of NO, which is an important effector of macrophage cytotoxicity, was measured based on the levels of its by-product NO_2. As shown in Figure 6 (A), NO_2 production in the 10-day treatment groups (100 and 200 mg/kg body weight p-KG03) increased 1.12-fold and 1.59-fold, respectively, in comparison with the untreated group, whereas the 20-day treatment groups did not show increases in NO_2 production. H_2O_2 production by macrophages that were cultured for 20 hours following stimulation with p-KG03 increased 1.16-fold and 1.27-fold (10-day treatment with 100 and 200 mg/kg body weight p-KG03, respectively), and 1.37-fold and 1.4-fold (20-day treatment with 100 and 200 mg/kg body weight p-KG03, respectively), as compared with the untreated group. H_2O_2 production increased with all of the treatments examined (Figure 6, B).

Discussion

Several parameters, such as NK cell activity, macrophage activity, antibody-dependent cellular cytotoxicity, induction of specific antibody, and T-cell

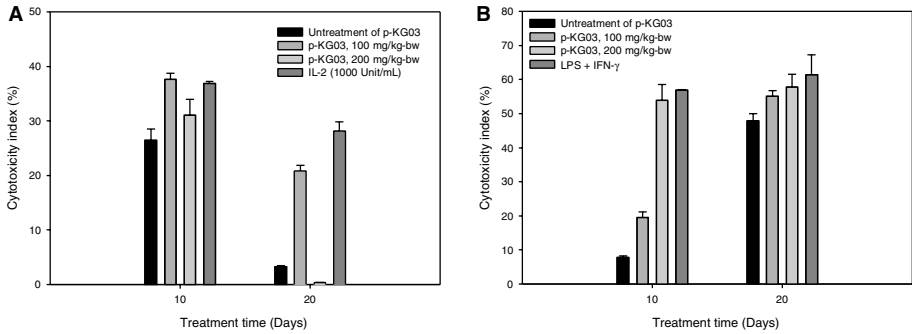

Fig. 4. Effects of p-KG03 on NK cell (**A**) and macrophage (**B**) cytotoxicity. Mice were orally administered the test compounds for 10 or 20 days. Natural killer cells and macrophage cytotoxicity levels were measured as described in the ''Materials and Methods'', and are expressed as the percentage of cytolysis of target cells. The results are presented as the means ± SE for 5 mice per group. The experiments were repeated twice.

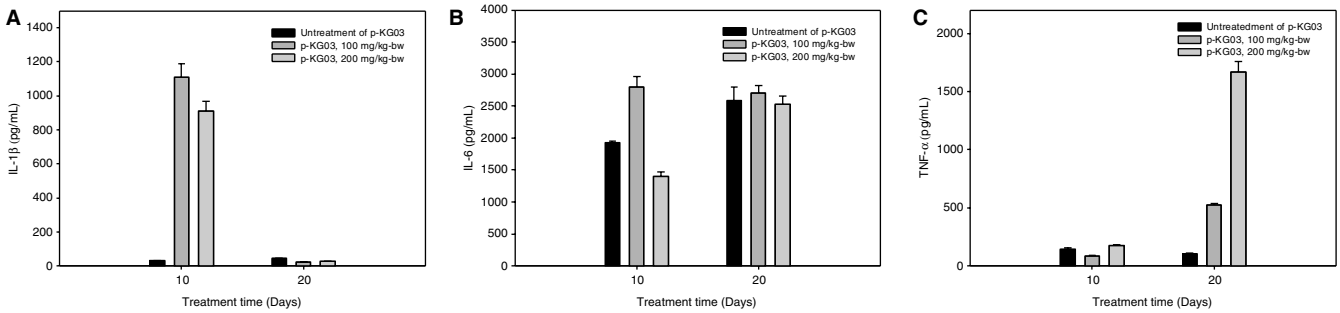

Fig. 5. Cytokine production by macrophages in response to p-KG03. Mice were orally administered the test compound for 10 or 20 days. Macrophages from control and p-KG03-treated mice were cultured for 20 hours. The culture supernatants were collected, and the levels of IL-1β (**A**), IL-6 (**B**), and TNF-α (**C**) were measured by ELISA. The results are presented as the means ± SE for 5 mice per group. The experiments were repeated twice.

and B-cell proliferation, may be used to evaluate the immunostimulatory activities of natural products (See et al., 1997; Benencia et al., 2000). Polysaccharides extracted from natural sources are known to potentiate immune functions and prevent tumor growth without direct cytotoxicity for tumor cells (Franz, 1989; Chihara, 1984). In particular, macrophages are known to produce and release various active products for the nonspecific primary defense against infectious agents (Nathan, 1977).

Macrophages are the major source of IL-1, TNF-α, IL-6, and IL-12, and they participate as major effector cells in resistance against infectious agents and tumor cells. Macrophage cytotoxicity is activated by a set of cytokine signals. Therefore, the number of peritoneal macrophages, the levels of NK cell activity, and various functions of murine peritoneal macrophages, such as antitumor activities and the production of TNF-α, NO, and H_2O_2, were assayed after exposure to p-KG03.

Since 100 and 200 mg/kg body weight were considered to be the appropriate concentrations of p-KG03 to induce the immune system without affecting the survival rate of the animals over 20 days, the mice were exposed to these regimens for 10 and 20 days before the measurement of macrophage function. With respect to these assays, we considered the possibility that the p-KG03 preparation used to stimulate the macrophages contained low levels of certain contaminants, such as endotoxin. To avoid interference with the interpretation of the results, p-KG03 was tested for the presence of endotoxin by the *Limulus amoebocyte* lysate assay. The endotoxin content of the p-KG03 preparation was below the lower limit of detection for this assay (<10 pg/ml).

The splenic PFC response against SRBC is one of the immune function parameters sensitive to immunomodulators. The increased number of peritoneal macrophages suggests that p-KG03 enhances the infiltration of macrophages into the peritoneal cavity and affects macrophage functions. In addition, p-KG03 increased polyclonal IgM and IgG antibody production.

Although the tumoricidal activities of NK cells increased after treatment, for 10 or 20 days, with 100 mg/kg body weight p-KG03, the NK cells of mice that were treated for 20 days with 200 mg/kg body

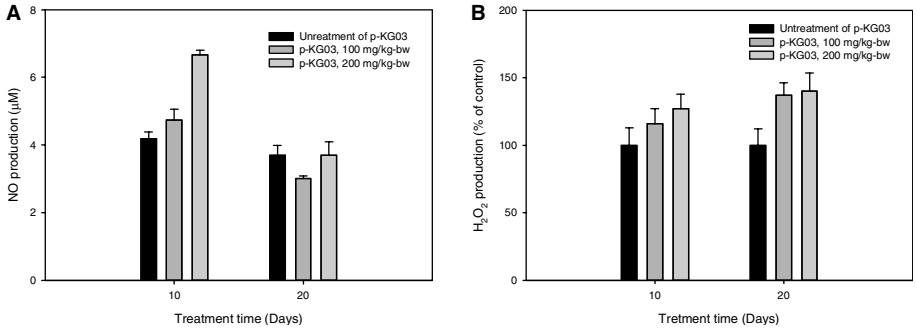

Fig. 6. Macrophage production of NO (**A**) and H_2O_2 (**B**) in response to p-KG03. Sulfated exopolysaccharide p-KG03 (100 and 200 mg/kg body weight) was orally administered to mice. Peritoneal macrophages were isolated and cultured for 20 hours. The culture supernatants were assayed for NO and H_2O_2. NO was measured as nitrite using the Griess reagent, and H_2O_2 production was measured fluorimetrically by horseradish-peroxidase-catalyzed oxidation of fluorescent scopoletin to nonfluorescent product (excitation at 350 nm, emission at 460 nm). The percentage of H_2O_2 production was obtained by comparison with the H_2O_2 level in the negative control group. The experiments were repeated twice.

weight p-KG03 did not show tumoricidal activity. NK-mediated cytotoxicity depends on the recognition and formation of NK-target conjugates and the secretion of NK-related cytokines, such as TNF-α (Lebow and Bonavida, 1990). TNF-α secretion is critical for the subsequent processes of NK activation and maturation driven by IL-2 (Jewett and Bonavida, 1994). In Figure 5 (C), p-KG03 clearly is effective at stimulating TNF-α, and it appears to induce tumoricidal activity. In particular, macrophages are known to produce and release various active products for nonspecific primary defense against foreign infectious agents. The tumoricidal activities of macrophages against B16 mouse melanoma cells increased after 10 and 20 days of treatment with p-KG03. The tumoricidal activities of the isolated macrophages increased in a dose-dependent manner in relation to all of the treatments examined. These results suggest that p-KG03 is stimulatory for macrophage functions. When p-KG03 was administered in vivo to mice for 10 and 20 days, the macrophage production levels of IL-1β, IL-6, and TNF-α increased. As demonstrated in Figure 5, p-KG03 induced dramatic increases in IL-1β and TNF-α production, whereas the levels of IL-6 were only slightly elevated. These results suggest that p-KG03 augments the production of these cytokines (TNF-α, IL-1, and IL-6). Since cytokines such as TNF-α, IL-1, and IL-6 are known to be potent macrophage activators as well as immunomodulatory agents, it seems likely that p-KG03 activates macrophages by upregulating the synthesis and production of these cytokines. Other polysaccharides, such as β-1,3-D-polyglucose derivatives, activate macrophages to induce the production of IL-1 (Rasmussen and Selhelid, 1989). In addition, polysaccharides from *Echinacea purpurea* increase the production by human macrophages of TNF-α, IL-1, IL-6, and IL-10 (Burger et al., 1997). Fucogalactan from *Grifola frondosa* elicits the release of TNF-α and NO from murine macrophages in vitro (Mizuno et al., 2000), and high molecular weight dextran sulfate (HMDS), which is a sulfated polysaccharide, is known to activate immune cells by inducing the production of IL-8 (Jagodzinski et al., 2002).

Further experiments were performed to measure the levels of NO and H_2O_2 released from macrophages. When p-KG03 was administered in vivo to mice for 10 and 20 days, the production levels of NO and H_2O_2 in the murine macrophages increased. TNF-α may facilitate the production of NO by binding to the upstream promoter response elements in the inducible nitric oxide synthase (iNOS) gene and by triggering iNOS gene transcription, as has been suggested previously (Lowenstein et al., 1993). Furthermore, NO has been identified as the major effector molecule in the destruction of tumor cells by activated macrophages (Duerksen-Hughes et al., 1992). On the basis of these results, it is conceivable that these cytokines act synergistically in macrophage cytotoxicity induced by p-KG03.

In conclusion, p-KG03 stimulates various functions of macrophages and NK cells, such as tumoricidal activities and the production of IL-1β, TNF-α, NO, and H_2O_2. However, further studies of the mechanisms and chemical structure of p-KG03 should be carried out in order to clarify the overall tumoricidal effects of this sulfated polysaccharide. The present study suggests that p-KG03 may have application as an agent in cancer immunotherapy.

Acknowledgments

This work was supported by grants to H.K.L. from the Development of Marine Novel Compounds Program of the Korean Ministry of Maritime Affairs and Fisheries and from the National Research Laboratory Program of the Korean Ministry of Science and Technology.

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