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Immunomodulating Activities of Polysaccharides Isolated from *Panax ginseng*

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ABSTRACT *Panax ginseng* C.A. Meyer has been traditionally used for the prevention and treatment of various chronic diseases and infections. Ginseng marc is a fibrous and insoluble by-product remaining after the extraction process of ginseng. In this research an extrusion process was employed to disintegrate the insoluble ginseng marc structure, and water-soluble ginseng marc polysaccharide (GMP) was isolated. GMP was examined for immunomodulatory effects in murine peritoneal macrophages. GMP significantly increased the lysosomal phosphatase activity and the phagocytic index of peritoneal macrophages ($P < .05$). The peritoneal macrophages treated with GMP also produced significantly more H_2O_2 and nitrite than the control without GMP treatment ($P < .05$). In addition, GMP (100 μg/mL) significantly increased the cell viability of peritoneal macrophages ($P < .05$). These results suggest that GMP is an effective nonspecific immunomodulatory agent, and its immunostimulating effects may be due to its ability to stimulate the production of reactive oxygen intermediates.

KEY WORDS: • *extrusion* • *ginseng* • *immunomodulation* • *macrophage* • *polysaccharides*

INTRODUCTION

PROCESSES TO REMOVE invading microbes and harmful foreign or endogenous substances are essential to maintain normal development and homeostasis in multicellular organisms. Macrophages are known to play an essential role in the host defense against primary infection and/or metastatic neoplasia.[1] Macrophages exert their tumoricidal activity not only by direct contact but also by the release of a number of cytotoxic/cytostatic factors such as tumor necrosis factor, interleukin-1, reactive oxygen species (ROS), and reactive nitrogen species (RNS).[2] Macrophages can be activated to become cytotoxic with a variety of agents that include several cytokines, bacterial-derived lipopolysaccharides (LPS), and various chemotherapeutic drugs.[3] It has been established that nitric oxide (NO) produced by macrophages is involved in the destruction of various intracellular pathogens as well as tumor cells, and places cells in cytostasis. Superoxide anion ($O_2^{\cdot -}$), H_2O_2, and other ROS are generated both enzymatically by oxidoreductases and nonenzymatically as the side products of reactions that utilize electron transfer. Mitochondria, the cytochrome P450s and their reductases, and nitric oxide synthase (NOS) have been suggested to generate ROS.[4]

Panax ginseng C.A. Meyer is a medicinal plant used widely as a nutraceutical source for the prevention and treatment of various chronic diseases and infections because of its strong physiological and pharmacological actions.[5] Certain fractions or purified individual ingredients of ginseng have been shown to exert cytotoxic or cytostatic activities against tumor cells.[6] Epidemiological studies have also demonstrated that ginseng intake is associated with a reduced risk of environmentally related cancers.[7] Polysaccharides isolated from ginseng root were found to have mitogenic,[8] hypoglycemic,[9] antitumor,[6] and macrophage cytokine-inducing[10] activities.

Ginseng marc occurs as a by-product of the manufacturing of ginseng extract. Presently, ginseng marc is either simply discarded or used in animal feed, although it contains a large amount of bioactive polysaccharides. Since ginseng marc consists of fibrous and insoluble constituents, utilization of ginseng marc requires an efficient solubilization process. The extrusion process, which combines high pressure, high shear, and heat, simultaneously performs mixing, cutting, crushing, pressing, expanding, drying, sterilizing, etc.[11] Extrusion has proven its engineering efficiency as a powerful tool to disintegrate the rigid cell wall matrix of plants. The extrusion process provides industrial advantages over the chemical processing, which is corrosive and produces undesirable waste effluents.[12,13] In this research, ginseng marc was processed by extrusion to disintegrate its insoluble structure without using chemicals.

The present study investigated the *in vitro* immunomodulatory effects of polysaccharides isolated from extruded ginseng marc, using murine peritoneal macrophages. The phagocytic activities as well as nitrite and H_2O_2 production in peritoneal macrophages were determined as biomarkers for immunomodulation.

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MATERIALS AND METHODS

Animals

Specific pathogen-free BALB/c mice were purchased from Samtaco Co. (Osan, Korea). The mice, which were 4–5 weeks of age, were acclimated for at least 1 week. All animals were maintained on lab chow (Samyang Co., Seoul, Korea) and tap water *ad libitum*. The animal quarters were strictly maintained at 22 ± 2°C and 50% relative humidity and followed a 12-hour light/dark cycle.

Extrusion of ginseng marc

Dried ginseng marc, the residue from the ethanol extraction process, was supplied from Koje Co. Ltd. (Chonan, Korea), ground to pass through a 600-μm sieve. Ginseng marc was extruded at a screw speed of 300 rpm, feed rate of 20 kg/hour, and moisture content of 25% using a twin screw extruder (DNDL-40, Bühler Co., Uzwil, Switzerland) with an *L/D* ratio of 20. The barrel was sectioned at a length of 176 mm and equipped with a jacket for heating/cooling. The ginseng residue was metered with a K-tron twin-screw volumetric feeder (K-tron Corp., Pitman, NJ). The screw configuration was $3 \times 1.5D$ forward + $2 \times 1D$ kneading disk + $3 \times 1D$ forward + $3 \times (1/3D$ reverse + $1/3D$ forward) + $4 \times 1D$ forward + $4 \times 0.75D$ forward element from the feed to the die. The die was a single 2-mm-diameter nozzle. The barrel temperature was maintained at 25, 50, 90, and 170°C, respectively, from the feeding zone to the die section throughout the experiments by circulation of cooling water or heating medium heated at the desired temperature through the jacket.

Fractionation of water-soluble ginseng marc polysaccharide (GMP)

The extruded ginseng marc (10 g) was solubilized in 200 mL of distilled water for 1 hour at 25°C, and centrifuged for 10 minutes at 6,500 *g*. The supernatant was filtered through filter paper, and 800 mL of isopropanol was added to the filtrate. The precipitate was washed with isopropanol and acetone, and dried polysaccharide was solubilized with distilled water to make a 1% solution, then centrifuged for 10 min at 6,500 *g,* and freeze-dried. The resulting polysaccharide was designated as GMP.

Characterization of GMP

Measurement of molecular weight was carried out by gel permeation chromatography (Alliance 2690, Waters, Milford, MA), eluting with 0.1 *M* $NaNO_3$ at a flow rate of 1.0 mL/minute and monitored by a refractive index detector (2410, Waters). The sugar composition was analyzed by a Bio-LC system (Dionex Co., Sunnyvale, CA) equipped with a Dionex Carbopac PA-100 column (4 × 250 mm), monitored by an electronic chemical detector and eluting with 22.6 m*M* NaOH at a flow rate of 0.3 mL/minute. Uronic acid content was estimated by the method of Blumenkrantz and Asboe-Hansen[14] and protein by the method of Bradford.[15] GMP contained arabinose (21.9%), galactose (22.6%), glucose (14.8%), rhamnose (5.8%), uronic acid (32.7%), and protein (2.2%). The average molecular mass of GMP was 12,380 daltons.

Isolation of peritoneal macrophage and culture conditions

Peritoneal macrophages were obtained from mice that had been injected intraperitoneally 3 days previously with 2 mL of thioglycolate broth (Becton Dickinson, Franklin Lakes, NJ). Following lavage of the peritoneal cavity with 6 mL of RPMI 1640 (GIBCO, Grand Island, NY), the cells were washed twice and resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Macrophages were seeded at the densities of 1×10^5 cells/well on a 96-well plate and allowed to adhere for 2 hours at 37°C in a 5% CO_2 humidified incubator. After a 2-hour incubation, nonadherent cells were removed by washing with the medium three times. More than 98% of the adherent cell population were macrophages according to the morphology and phagocytic criteria.[16] GMP was dissolved in Dulbecco's phosphate-buffered saline (DPBS) and filtered (pore size, 0.22 μm). Macrophages were exposed to either GMP (100 μg/mL) or 20 μg/mL LPS (from *Escherichia coli* serotype, Sigma, St. Louis, MO) and incubated for 48 hours in a humidified incubator containing 5% CO_2 at 37°C.

Assay of macrophage lysosomal phosphatase activity

Lysosomal phosphatase activity in peritoneal macrophages was determined using *p*-nitrophenyl phosphate as a substrate according to the method of Park *et al*.[17] The activated macrophage monolayer was centrifuged at 1,500 rpm for 10 minutes, and the supernatant was removed. The cell layer was washed with RPMI 1640 containing 10% FBS and solubilized with 0.1% Triton X-100. The microtitre plate was then incubated with 200 μL of *p*-nitrophenyl phosphate liquid substrate (Sigma) in a humidified incubator containing 5% CO_2 at 37°C for 30 minutes. The reaction was stopped by adding of 50 μL of 0.2 *M* borate buffer (pH 9.8), and the absorbance was measured at 405 nm using an ELISA reader (Molecular Devices Co., Palo Alto, CA).

Phagocytic index

Phagocytosis was examined as described by Yoshida *et al*.[18] The assays for phagocytosis were performed in well chamber slides (Lab-Tek 8 Chamber slide with cover, Nalgene Nunc International, Roskilde, Denmark) by incubating 10^5 macrophages with latex beads at a ratio of 10 beads/cell for 1 hour at 37°C. Phagocytosis was terminated by washing the wells five times with RPMI 1640 with 10% FBS. The cells on the coverslip were treated with methyl alcohol and stained with May-Grunwald Giemsa. The phagocytic index (% of control based on the number of macrophages

phagocytizing six or more beads) was determined by microscopic examination.

Measurement of nitrite

Nitrite production, an indicator of NO synthesis, was measured in the culture supernatant of macrophages, as previously described by Kleinerman *et al*.[19] Briefly, after mixing 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) with 100 μL of culture supernatant, optical density at 540 nm was measured by using a microplate reader (Molecular Devices). Nitrite concentrations were calculated from a standard curve derived from sodium nitrite dilutions in culture medium.

Assay for H_2O_2

Secretion of H_2O_2 was fluorimetrically measured by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin to a nonfluorescent product.[20] The 460 nm emission from reduced scopoletin, when excited by light at 350 nm, is extinguished when scopoletin is oxidized by H_2O_2 in the presence of horseradish peroxidase. Under the assay conditions, the loss of fluorescence was proportional to the concentration of H_2O_2.

Cell viability test

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent.[21] After treatment of the samples on a 96-well plate culture, the cells were washed twice with DPBS and incubated with 110 μL of 0.5 mg/mL MTT for 2 hours at 37°C. The medium was discarded, 100 μL of dimethyl sulfoxide was then added after a 30-minute incubation, and absorbance at 570 nm was measured by using a microplate reader.

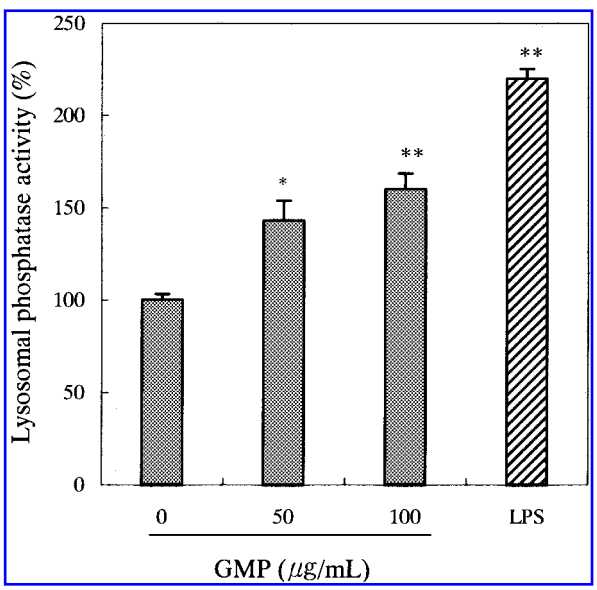

FIG. 1. Effect of GMP on lysosomal phosphatase activity of peritoneal macrophages. Macrophages were treated with doses of 50 and 100 μg/mL GMP and 20 μg/mL LPS for 48 hours. Data shown are mean ± SEM of the results of six cultures, expressed as a percentage of the control. The control value was 1.46 ± 0.05 μM *p*-nitrophenol released/30 minutes/10^5 cells. $*P < .05$, $**P < .01$; significantly different from the control (no treatment).

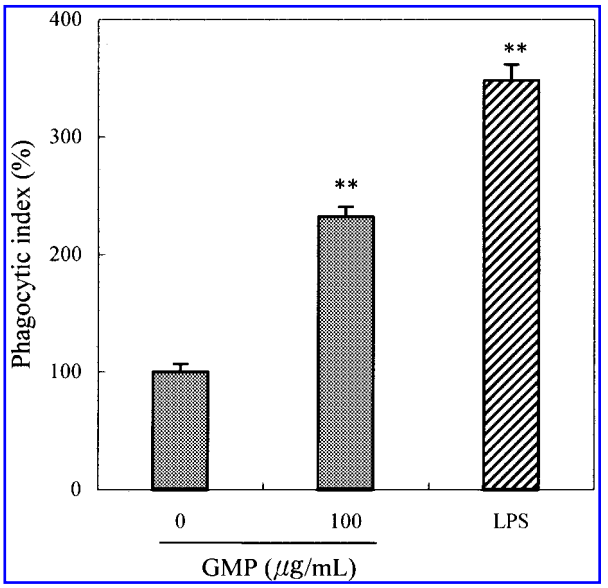

FIG. 2. Effect of GMP on phagocytic index of peritoneal macrophages. Macrophages were treated with doses of 100 μg/mL GMP and 20 μg/mL LPS for 48 hours. Data shown are mean ± SEM of the results of six cultures, expressed as a percentage of the control. The control value was 60 ± 4.04. $**P < .01$; significantly different from the control (no treatment).

Statistical analysis

All experiments were performed four times, and the results are expressed as mean ± SE. Statistical differences between groups were determined by one-way analysis of variance using an SAS computer program (SAS Institute, Cary, NC). Significant differences are indicated ($P < .05$).

RESULTS

Effect of GMP on lysosomal enzyme activity of peritoneal macrophages

The immunomodulatory effects of polysaccharide isolated from ginseng marc (GMP) were monitored through the changes in peritoneal macrophage function. Figure 1 outlines the effect of GMP on the lysosomal enzyme activity of the peritoneal macrophages. Phosphatase activity stimulated by LPS (20 μg/mL) was 220% higher than that in the control group ($P < .05$). The lysosomal enzyme activity of the peritoneal macrophages stimulated by GMP was significantly increased, by 142% at 50 μg/mL and 160% at 100 μg/mL, compared with the control group ($P < .05$).

Effect of GMP on phagocytosis index of peritoneal macrophages

One of the important functions of macrophages is to ingest and destroy the inhaled particulate matter by phagocytosis. The phagocytosis indices of peritoneal macrophages cultured for 48 hours are shown in Fig. 2. Phagocytosis stimulated by LPS (20 μg/mL) was 348% higher than that in the control group ($P < .05$). The phagocytosis index of macrophages treated with GMP at a dose of 100 μg/mL was 232% higher than that in the control group ($P < .05$).

Effect of GMP on nitrite production of peritoneal macrophages

The effect of GMP on peritoneal macrophages function was also assessed through changes in the release of reactive oxygen intermediates (ROIs) (NO and H_2O_2). As shown in Fig. 3, production of nitrite by macrophages stimulated by LPS (20 μg/mL) was 408% higher than that in the control group. Administration of GMP significantly increased nitrite production of macrophages, by 244% at 50 μg/mL and 269% at 100 μg/mL, compared with the control group.

Effect of GMP on H_2O_2 production of peritoneal macrophages

H_2O_2 production of the macrophages is shown in Fig. 4. Production of H_2O_2 by macrophages stimulated by LPS (20 μg/mL) was 167% higher than that in the control group. GMP significantly increased H_2O_2 production of the macrophages, by 131% at 50 μg/mL and 139% at 100 μg/mL, compared with the control group.

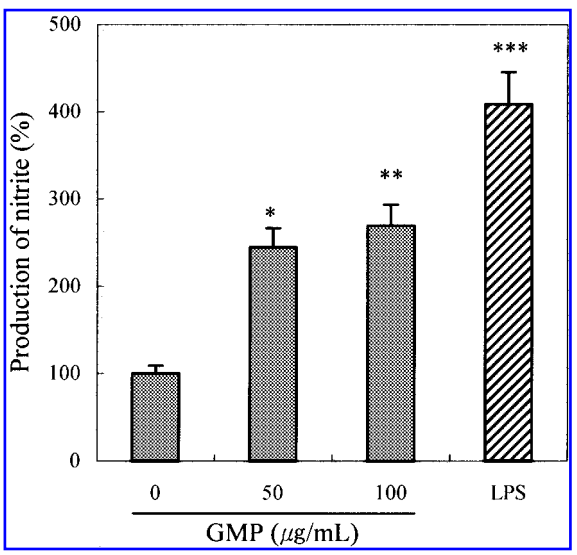

FIG. 3. Effect of GMP on nitrite production of peritoneal macrophages. Macrophages were treated with doses of 50 and 100 μg/mL GMP and 20 μg/mL LPS for 48 hours. Data shown are mean ± SEM of the results of six cultures, expressed as a percentage of the control. The control value was 0.07 ± 0.01 nmol/10^5 cells. *$P < .05$, **$P < .01$, ***$P < .001$; significantly different from the control (no treatment).

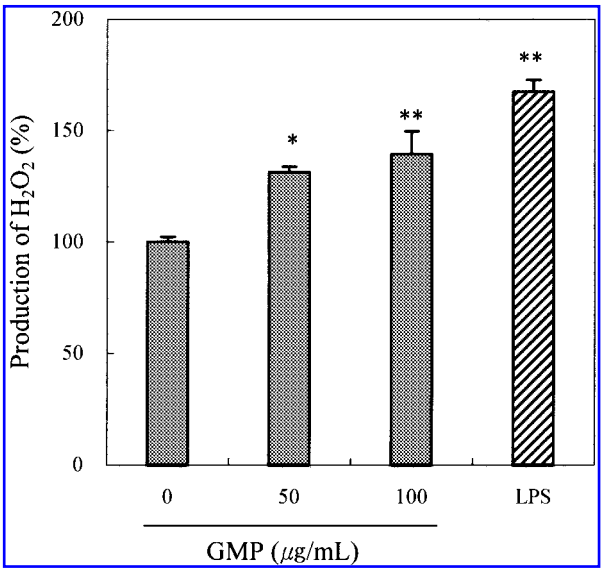

FIG. 4. Effect of GMP on H_2O_2 production of peritoneal macrophages. Macrophages were treated with doses of 50 and 100 μg/mL GMP and 20 μg/mL LPS for 48 hours. Data shown are mean ± SEM of the results of six cultures, expressed as a percentage of the control. The control value was 0.92 ± 0.01 nmol/10^5 cells. *$P < .05$, **$P < .01$; significantly different from the control (no treatment).

Effect of GMP on survival of peritoneal macrophages

The effects on the growth stimulation index are shown in Fig. 5. The survival of peritoneal macrophages was inhibited in LPS-treated macrophages, but significantly enhanced in GMP-treated macrophages ($P < .05$). The growth index of macrophages treated with GMP at a dose of 100 μg/mL was 133% than that of the control group.

DISCUSSION

There are many approaches to the study of immunomodulation; we have chosen to focus on macrophages because of their pivotal role in immune system function. Macrophages are integral to disease resistance since they engage in phagocytosis and the destruction of pathogens via the production of ROS and RNS, lysozyme, and other chemical mediators. It is well known that free radicals, including ROS, may react with several biomolecules, via nucleic acids, lipids, carbohydrates, and proteins, and in doing so damage the cells. These perturbations are believed to underlie tissue injuries and physiological disorders.[22,23]

The peritoneal macrophage functions *in vitro* were assessed through the phagocytic response and through the production of ROIs after activation with GMP. In this study, the function of the elimination stage of the phagocytic process, measured by the lysosomal enzyme activity (Fig.

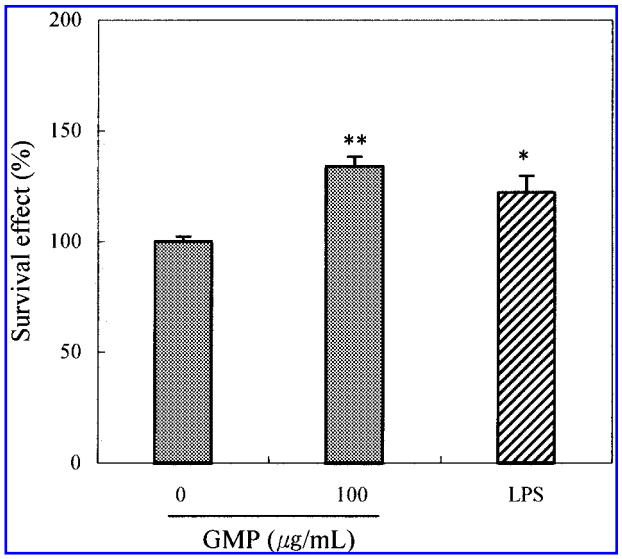

FIG. 5. Effect of GMP on viability of peritoneal macrophages. Macrophages were treated with doses of 100 μg/mL GMP and 20 μg/mL LPS for 48 hours. Data shown are mean ± SEM of the results of six cultures, expressed as a percentage of the control. The control value was 0.58 ± 0.003 OD. *$P < .05$, **$P < .01$; significantly different from the control (no treatment).

1) and phagocytic index (Fig. 2) in peritoneal macrophages treated with GMP, was significantly higher than that in the control group ($P < .05$). This result suggests that GMP activates the macrophages through modulation of lysosomal enzyme activity in peritoneal macrophages, and also affects partially the ability of lysosomal enzyme in peritoneal macrophages to respond appropriately to foreign agents. Thus, the overall percentage of macrophages eliciting the phagocytic responses as well as the capacity of individual cells to ingest foreign particles was increased.

When macrophages are stimulated with foreign agents, a variety of chemicals, cytokines, and chemokines are released to induce functional defense systems.[24] Macrophages, in addition to eliciting a phagocytic response to a pathogen, produce a variety of ROIs such as $O_2^{\cdot -}$, H_2O_2, and NO to regulate their own activity and the activity of the other immune cells.[25] It has been suggested that NO is a cytotoxic effector molecule of macrophage-mediated tumoricidal and bactericidal actions,[26,27] and murine peritoneal macrophages in culture synthesize significant quantities of NO and H_2O_2 in response to LPS.[28] Therefore, the present study also determined the effects of GMP on the peritoneal macrophages' ability to produce ROIs. As shown in Fig. 3, administration of GMP significantly increased NO_2^- production by peritoneal macrophages in a dose-dependent manner ($P < .05$). The increase in NO production by the GMPs is also an important observation of the present study, since the release of NO by macrophages has been known to be the predominant mechanism by which infectious agents are destroyed.[29] The GMP also induced a dose-related increase in H_2O_2 by the peritoneal macrophages (Fig. 4). The ability of GMP to induce H_2O_2, as illustrated in this study, may contribute to the ability of ginseng to produce the early immune response mediators and to have antiviral effects. These immunomodulatory properties of GMP could be associated with its claimed prophylactic and therapeutic effects against the common cold.[30] Humans lacking functional NADPH oxidase producing $O_2^{\cdot -}$ suffer from chronic infections that result in high mortality.[31] Hence, the stimulation of macrophage activity could increase the effective destruction of pathogens, leading to prevention of chronic infection. Although we did not measure the macrophage-mediated cytotoxicity in this study, it is speculated that the ingestion of GMP has antitumor and bactericidal effects through ROI-producing mechanisms.

Macrophages can also contribute to tissue damage when activated inappropriately.[32] Therefore, significant modulation of macrophage activity can be viewed as deleterious. The inappropriate generation of ROS by macrophages can result in localized tissue damage and has been implicated in the progression of autoimmune diseases.[33] However, in the present study, the survival of peritoneal macrophages treated with GMP (50 μg/mL) was significantly increased compared with the control group (Fig. 5). In human polymorphonuclear cells, glutathione plays an important role in protection from auto-oxidation.[34] Glutathione interacts strongly with hypochlorous acid (HOCl), forming small sulfonamides and thiosulfonates.[35] In mammalian macrophages, glutathione production has been shown to be up-regulated following treatment with pro-oxidants and viruses,[36] illustrating that glutathione is used both to protect and to regulate mammalian phagocytes. Both catalase and superoxide dismutase confer protection against ROS, but may have other protective roles as well. Metallothionein, a small inducible protein containing numerous cysteinyl sulfhydryls, is known for its protective effects against metals, as well as its ability to scavenge ROS.[37] Metallothionein may also shield cells against oxidative stress resulting from exposure to organic compounds.[38] That macrophages are well protected against oxidative stress could be due to the specific characteristics of their membranes, or the presence of additional antioxidants.[39]

In conclusion, we have demonstrated that GMP results in an augmentation of macrophage function and is a potent stimulator of the release of cytotoxic mediators. These results suggest that GMP is an effective nonspecific immunomodulatory agent, and that the immunostimulating effects of ginseng polysaccharides may be due to their stimulating the ROI-producing mechanism. Clearly, studies need to be done to evaluate the *in vivo* effects of immunostimulatory GMP on both the respiratory burst and antimicrobial defenses in macrophages.

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