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Characteristic immunostimulation by MAP, a polysaccharide isolated from the mucus of the loach, *Misgurnus anguillicaudatus*

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Abstract

To evaluate the mechanism for the biological activity of a natural polysaccharide isolated from the mucus of the loach, *Misgurnus anguillicudatus* (MAP), the immunomodulatory of MAP was investigated by the methods of molecular biology and cellular biology. The results showed that MAP enhanced proliferation of T lymphocyte, IL-2 expression of Th1 cells, and IL-4 expression of Th2 cells. Time dependence of the secretion of cytokines showed that Th1 cell was the primary cellular target affected by MAP on T lymphocyte. However, MAP did not increase directly the proliferation of B cells and enhanced less IgM antibody production. Moreover, MAP improved the viability of peritoneal macrophages, stimulated TNF-α and IL-6 production and induced the inducible nitric oxide synthase (iNOS) transcription in macrophages. In addition, MAP exerted its immunomodulating activity at an optimal dose of 30 μg/ml. At this concentration, MAP promoted farthest proliferation of spleen lymphocyte and macrophages. Consequently, MAP enhanced the immune system functions. In conclusion, the biological activity of the loach, which was as traditional Chinese medicine in folk remedies for the treatments of hepatitis, osteomyelitis, carbuncles, inflammations and cancers, as well as for the restoration to health in debilities by various pathogens and aging, may mainly result from MAP selectively activating T cells and macrophages and stimulating secretion of some cytokines.

Keywords: Polysaccharide; Loach; Immunomodulatory; T cells; B cells; Macrophages

1. Introduction

Many polysaccharides and polysaccharide–protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants, and their biological activities have attracted more attention recently in the biochemical and medical areas due to their immunomodulatory and anti-cancer effects (Ooi & Liu, 2000). The great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells. In view of the need for new anti-cancer compounds with low toxic potential, numerous polysaccharides from different biological origins have been investigated for antitumor and immunomodulating activities (Han et al., 2001; Kim et al., 1996a). It is generally accepted that polysaccharides enhance various immune responses in vivo and in vitro. In many oriental countries, several immunoceuticals composed of polysaccharides have been accepted such as lentinan, schizophyllan and krestin (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Liu, Ooi, & Fung, 1999).

The loach (*Misgurnus anguillicaudatus*) has long been employed as traditional Chinese medicine in folk remedies for the treatment of hepatitis, osteomyeitis, carbuncles, inflammations and cancers, as well as for the restoration to health in debilities caused by various pathogens and aging (Qin, Huang, & Xu, 2002a). Some vertebrate lectins, purified from the skin mucus or egg of the loach, were found to induce release of cytotoxin from fresh murine bone narrow cells or macrophages and lyse tumor cells but not normal spleen cells (Goto-Nance & Watanabe, 1995; Okutumi, Nakajima, Sakakibara, Kawauchi, & Yamazaki, 1987). A novel antimicrobial peptide named misgurin, which consists of 21 amino acid residues, was isolated and identified from the loach (Park, Lee, Park, Kim, & Kim, 1997).

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A deaminated neuraminic acid-containing glycoprotein from the skin mucus of the loach, was isolated and characterized (Mariko, Yoichiro, Toshhisa, & Makio, 1994). Our group isolated polysaccharide from the mucus of the loach *Misgurnus anguillicaudatus* (MAP), a kind of neutral free polysaccharide, and MAP were shown to exhibit a variety of pharmacological properties including anti-inflammatory, anti-oxidation, hypoglycemic and anti-hyperlipidemia activates, and enhance the immune system (Qin et al., 2002a; Qin, Huang, & Xu, 2002b).

In the present study, we investigated the effects of MAP on the immune system at the cellular level, and demonstrated that this polysaccharide selectively activated directly T cells and macrophages, but not B cells.

2. Materials and methods

2.1. Materials

Specific pathogen free (SPF) BDF1 mice (female, 5–7 weeks old) were obtained from Hubei Provincial Center of Medical Experiment, P.R. China. They are maintained under SPF conditions until used. Standard rodent food and water were supplied. RPMI 1640 medium was purchased from GIBCO BRL (Grand Island, NY, USA). Medium was supplemented with 10% fetal calf serum (FCS, GIBCO) and 50 μM 2-mercaptoethanol (Sigma, St Louis, MO, USA).

Loach (*Misgurnus anguilliacaudatus*, weight 8 ± 1.5 g, length 8.5 ± 5 cm) was purchased from market in Wuhan City, China. *Misgurnus anguilliacaudatus* polysaccharide (MAP) was isolated and purified as described by Qin et al. (2002b). We further identified that its average molecular weight was 130 kDa by gel permeation chromatography (GPC); the major structure monomers of MAP were composed of D-galactose, L-fucose and D-mannose by gas chromatography (GC) and paper chromatography (PC); the monomers link each other by α-1, 3 bonds through Smith degradation test. These data are same to our previous results (Qin et al., 2002b).

2.2. Preparation of cells

Splenic lymphocytes. Total splenocyte populations were prepared from mice as described by Kim et al. (1996b). The cells were freed of red blood cells by treatment with lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, and 0.1 mM Na_2 EDTA, pH 7.4). To remove adherent cells such as macrophages, total spleen cells were incubated for 2 h in petri dishes at a concentration of 5×10^6 cells/ml. The suspended cell populations were collected and used as the splenic lymphocyte populations.

B cells and T cells were prepared from splenic single suspensions using the nylon-wool method (Sun & Wang, 1999). Briefly, T cells were separated over nylon wool columns and B cells were eluted from the nylon wool column with cold PBS. B cells were further purified by incubating the eluted cells with a cocktail of antibodies specific for T cells (anti-CD4, anti-CD8, and anti-Thy1.2) and the T cell fractions were purified by depleting B cells with anti-I-Ad and TIB 120 (anti-HSA) followed by complement depletion. The purity of the respective cell preparations was >93% as determined by FACS analysis. Antibodies were purchased from PharMingen (San Diego, CA).

Cells were cultured at $1–5 \times 10^6$ cells/ml in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, glutamine and 2-mercaptoethanol.

Peritoneal macrophages. Peritoneal macrophages were isolated from the abdominal cavity as described by Klimetzek and Remold (1980). Briefly, peritoneal cells were seeded at a density of $5–6 \times 10^5$ cells/cm^2 on Teflon-coated petri dishes (100×15 mm) and the macrophages were allowed to adhere for 2–3 h at 37 °C in 5% CO_2 humidified atmosphere. The non-adhered cells were removed and cold PBS (15 ml) containing 1.5% FCS was added, followed by 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were harvested. Macrophages were cultured in RPMI 1640 supplement with 10% fetal calf serum, 2 mM 2-mercaptoethanol, 100 U/ml penicillin and streptomycin (100 μg/ml).

2.3. Proliferation assay

MAP was added to the above cells at concentrations of 1–100 μg/ml on day 0. Specific lymphocyte mitogens, such as concanavalin A (Con A, T cell mitogen) and lipopolysaccharide (LPS, B cell mitogen) were used for reference purposes at a final concentration of 5 μg/ml. The proliferation of the cells was examined by using 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) (Mosmann, 1983), which is based on the ability of mitochondrial enzyme, succinate dehydrogenase to cleave MTT to the blue compound formazan. The cells were incubated for the periods indicated in 2 ml of medium containing agents. Then 400 μl of MTT solution in phosphate buffered saline (PBS) (5 mg/ml, pH 7.4) was added to each well and the cells were further incubated for 4 h at 37 °C and 5% CO_2 tension. Then 1 ml of 0.04 N HCl solution in isopropanol was added and the cells were sonicated at 10% maximum power for 3 s and the resultant colored product was read on a Microplate Reader (MPR.A4i II, Tosoh, Tokyo, Japan) at 570 nm.

2.4. Antibody production of B cell

Elevated IgM levels were used to determine polyclonal B cell activation. Spleen cells were cultured with MAP (1–100 μg/ml) for 72 h. Antibody forming cells (AFCs) were counted using the plaque forming cell (PFC) assay, as described previously (Han et al., 1996, 1998). The PFC assay involved mixing immunized cells with complement,

trinitrophenyl-conjugated SRBC, and agarose (350 μl), plating the mixture on a petri dish and incubating for 2 h in a CO_2 incubator. The number of plaques was counted and expressed as AFCs/10^6 cells.

2.5. Cytokine gene expression

RT-PCR was performed to determine changes in cytokine gene expression. The spleen cells or macrophages, cultured in the presence or absence MAP for the periods indicated, were lysed by TRIzol reagent (Gibco BRL), and the preparation of total RNA from the lysate was performed according to the supplier's instruction manuals.

Reverse transcription (RT) of the RNA was performed using a GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ, USA) with 100 ng of total cellular RNA. The mixture was incubated for 1 h at 37 °C, and for 5 min at 99 °C. PCR was then carried out with 2.5 units of AmpliTaq DNA polymerase and 10 pmol of cytokine primers for IL-2, IL-4, IL-6, IFN-γ and TNF-α in the same tube. The sequences of the primers used were as follows: for IL-2, sense 5′-CTT GCC CAA GCA GGC CAC AG-3′, anti-sense 5′-GAG CCT TAT GTG TTG TAA GC-3′; for IL-4, sense 5′-GAA TGT ACC AGG AGC CAT ATC-3′, anti-sense 5′-CTC AGT ACT ACG AGT AAT CCA-3′; for IL-6, sense 5′-TGC TGG TGA CAA CCA CGG CC-3′, anti-sense 5′-GTA CTC CAG AAG ACC AGA GG-3′; for IFN-γ, sense 5′-AGC GGC TGA CTG AAC TCA GAT TGT AG-3′, anti-sense 5′-GTC ACA GTT TTC AGC TGT ATA GGG-3′; for TNF-α, sense 5′-CCT GTA GCC CAC GTC GTA GC-3′, anti-sense 5′-TTG ACC TCA GCG CTG AGT TG-3′; for β-actin, sense 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, anti-sense 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. PCR was performed in a Bio-Rad Cycler (Bio-Rad. Lab., Richmond, CA, USA) starting with a 5 min incubation step at 95 °C, then 30 cycles of: 53 s at 94 °C, 1 min at 53 °C, and 2 min at 72 °C, followed by a 7 min incubation step at 72 °C to complete the polymerization. PCR products were electrophoresed on a 2% agarose gel and photographed after staining with ethidium bromide.

2.6. Macrophages iNOS gene expression and nitrite quantification

Inducible nitric oxide synthase (iNOS) gene expression level was determined by RT-PCR using the primers: sense 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′ and antisense 5′-GGG AGT AGC CTG TGT GCA CCT GGA A-3′ Peritoneal macrophages were plated and incubated 5×10^5 cells/ml in 24-well culture plates and cultured in the presence or absence MAP for 24 h in the presence of 5% CO_2 at 37 °C. Cells were lysed by TRIzol reagent and preparation of total RNA from the lysate was subject to RT-PCR. The accumulation of nitrite in the supernatants, which was used as an indicator of NO production in the medium, was measured by the method of Ding, Nathan, and Stuehr (1988). The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Nitrite production was determined by measuring absorbance at 550 nm versus an $NaNO_2$ based standard curve.

3. Results

3.1. Effect on the functions of spleen lymphocytes

As a first step towards understanding the immunomodulatory activity of MAP, the effect of MAP on four kinds of cell populations i.e., total spleen cells, macrophage-depleted cells, B cells, or T cells proliferation was investigated. As shown in Fig. 1, MAP was found to significantly increase the proliferation of total or macrophage-depleted cell populations and more strongly increases that of T cells. However, MAP had less influence on the proliferation of B cells. Moreover, MAP exhibits optimal bioactivities at the dose of 30 μg/ml, presenting immuno-reagent manner. Next, the mode of action of MAP was compared with other lymphocyte mitogens in three different preparations of lymphocytes. T cell mitogens (PHA and Con A), B cell mitogen (LPS), and comitogen (PWM) showed their characteristic action spectra to lymphocyte subsets. Similarly to T cell-responsive mitogen (PHA and ConA), MAP showed mitogenicity on T cell populations, but not on B cell populations (Fig. 2). Although, at the concentration of 30 μg/ml, MAP increased trivially the proliferation of B cell populations, this might have resulted from the small proportion of contaminating T cells present.

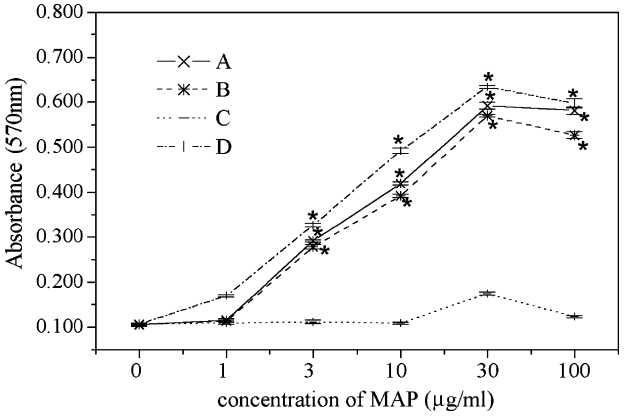

Fig. 1. The effect of MAP on proliferation of spleen cells. MAP was administered to four kinds of cell preparations. Total cell populations (A), macrophage-depleted cell populations (B), B cell populations (C), T cell populations (D). MAP was given at concentrations from 1 to 100 μg/ml for 72 h. After incubation, the degree of cells proliferation was measured by MTT assay. The data presents the mean values ± standard derivation of three independent experiments, which were done in six separate analyses. Significance was determined using the Student's *t*-test versus the control group (*$P<0.01$).

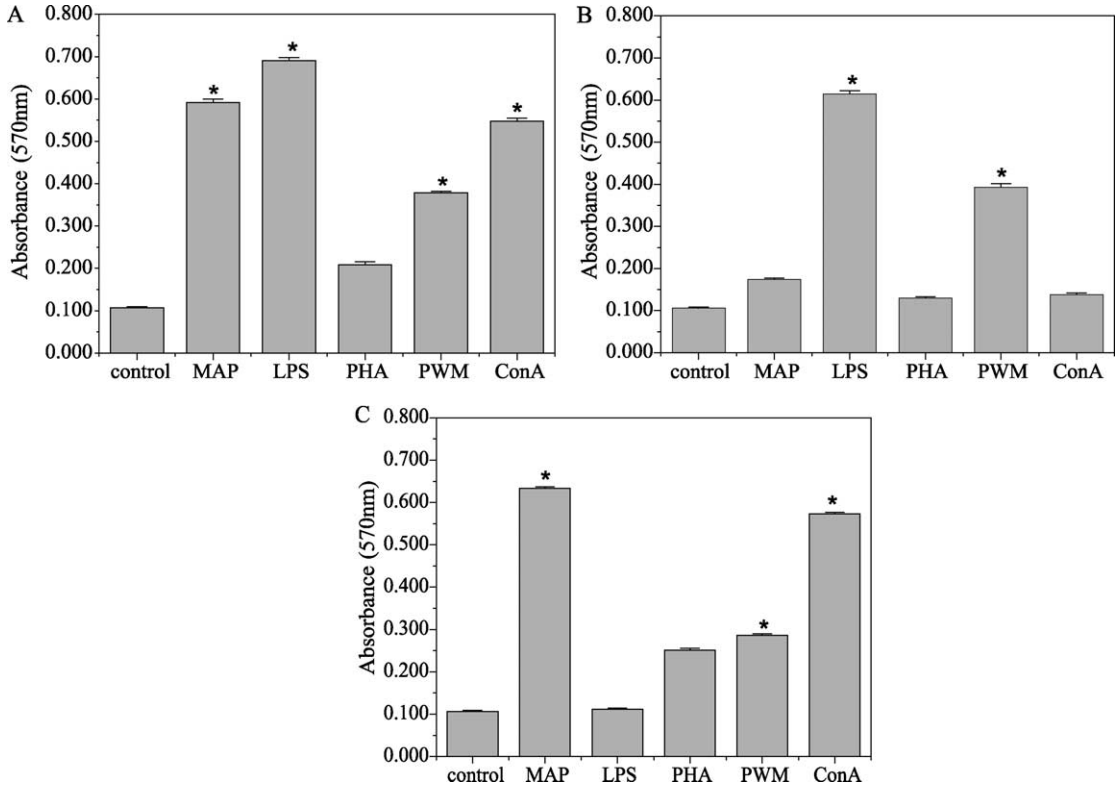

Fig. 2. Comparison of mitogenic pattern of MAP with specific mitogens. MAP was given to three different cell populations such as total cell populations (A), B cell populations (B), and T cell populations (C). Each cell population was treated with 30 μg/ml MAP or one of the various mitogens such as concanavalin A (Con A), pokeweed mitogen (PWM), lipopolysaccharide (LPS), and phytohemagglutinin (PHA) at 5 μg/ml for 72 h. After incubation, the degree of the lymphocyte proliferation was measured by the MTT assay. The data presents the mean values ± standard deviation of three independent experiments, which were done in six separate analyses. Significance was determined using the Student's *t*-test versus the control group (*$P < 0.01$).

The effects of MAP on T cells and B cells functions were further investigated to confirm above results that MAP activates T cells, but not B cells. T cells are characterized as either CD8+ or CD4+ T cells on the basis of their functional capabilities and cytokine profiles. Among these, CD4+ Th1 cells secrete cytokines, such as IL-2, and are associated with cell-mediated immune responses, and CD4+ Th2 cells produce IL-4, which helps B cells proliferate and differentiate. In the present study, splenic lymphocytes were incubated with MAP for 24 h and the expressions of IL-2 and IL-4 mRNAs were determined. As shown in Fig. 3, MAP increased the gene expressions of mRNA of IL-2 and IL-4, resembling with T cell activator Con A. The effects of MAP on B cells functions were further examined by determining the increased level of polyclonal IgM antibody production in B cells, and MAP was found to activate B cells slightly (Fig. 4). The above-mentioned results suggest that MAP selectively activates T cells, but does not affect B cells directly.

3.2. Effect on the cytokine mRNA expression of T lymphocytes

T lymphocytes isolated from splenic cells were incubated with MAP up to 48 h and the mRNA expression of cytokines such as IL-2, IL-4, IL-6 and IFN-γ were determined (Fig. 5). IL-2 mRNA expression was observed at the early time point of 3 h, and was increased in a time-dependence manner in MAP-treated T lymphocytes. IFN-γ mRNA was expressed strongly by the treatment of MAP from 3 h and downregulated thereafter. The level of IL-6 mRNA expression was slightly enhanced from the time point of 24 h by the treatment of MAP. IL-4 mRNA expression was detected from the time point of 48 h, when T lymphocytes were treated with MAP.

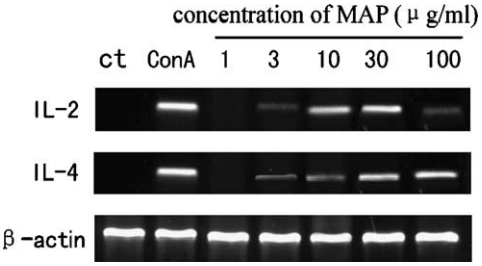

Fig. 3. The effect of MAP on cytokine IL-2 and IL-4 mRNA expression in splenocytes. Spleen cells (5×10^5 cells/ml) were incubated with MAP at concentration of 1–100 μg/ml or Con A (5 μg/ml) for 48 h. After incubation, total RNA was isolated and the mRNA expression levels of IL-2 and IL-4 were determined by RT-PCR. UN, chemically untreated control group.

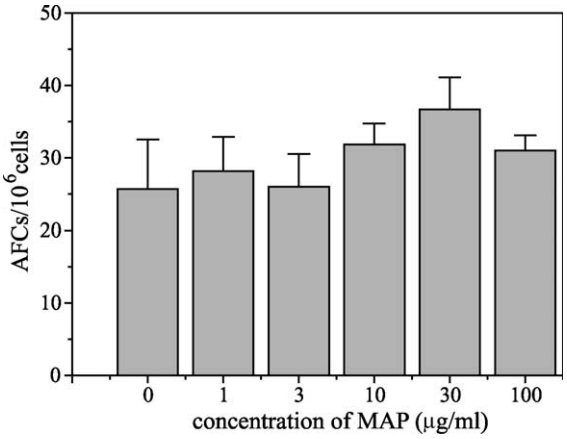

Fig. 4. The effect of MAP on the in vitro polyclonal IgM antibody production of B cells. Spleen cells (1×10^6 cells/ml) were activated with MAP at concentrations of 1–100 μg/ml for 72 h, and polyclonal IgM antibody production by B cells was determined by PFC assay. Significance was determined using Student's *t*-test versus the control group ($^*P<0.01$).

3.3. Effect of MAP on macrophages viability

Peritoneal macrophages were incubated with MAP at concentrations from 1 to 100 μg /ml for 72 h. As shown in Fig. 6, MAP increased proliferation of macrophages. Especially, at the concentration of 30 μg/ml, proliferation activity of MAP on macrophages was up to maximum. It was demonstrated that MAP possessed the typical characteristic of immunomodulatory agents.

3.4. MAP increased the cytokine mRNA expression of peritoneal macrophages

The effect of MAP on the expression and production of TNF-α and IL-6 were investigated. Macrophages were cultured with MAP at the dose of 30 μg/ml and RNA was isolated after 3, 6, 12, 24 and 48 h incubation. Peritoneal macrophages were also stimulated for 48 h with LPS

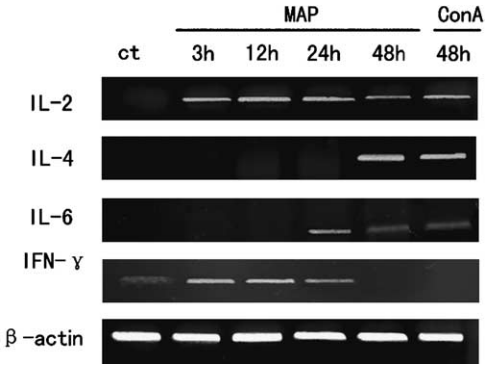

Fig. 5. Effect of MAP on cytokine specific mRNA expression produced by T lymphocytes. T lymphocytes isolated from splenic cells were cultivated with MAP at a final concentration of 30 μg/ml, and T cells untreated with MAP are control. After incubation up to 48 h, total RNA was isolated and level of mRNA expression of IL-2, IL-4, IL-6, and IFN-γ was evaluated by RT-PCR. RT-PCR product from T cells treated with ConA (5 μg/ml) was used as a positive control.

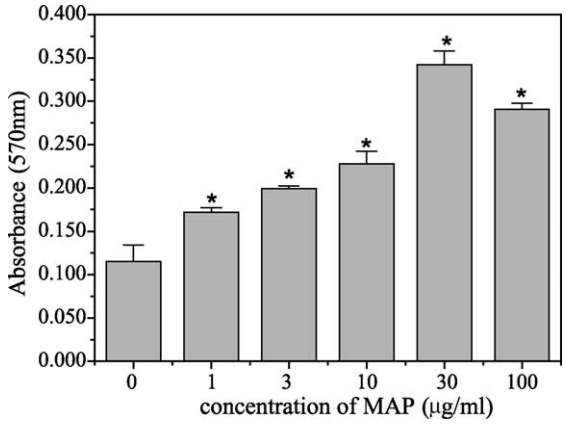

Fig. 6. The effect of MAP on proliferation of mice peritoneal macrophages. Macrophages (1×10^5cells/ml) were cultivated with MAP at different concentrations from 1 to 100 μg /ml for 72 h. After incubation, the degree of cells proliferation was measured by MTT assay. The data presents the mean values ± standard derivation of three independent experiments, which were done in six separate analyses. Significance was determined using the Student's *t*-test versus the control group ($^*P<0.01$).

(5 μg /ml). The expression of cytokines were determined by RT-PCR and analyzed on agarose gel electrophoresis. As shown in Fig. 7, TNF-α mRNA expression was enhanced at 3 h after MAP treatment and then slowly reduced to control level at 48 h. IL-6 mRNA was expressed strongly after macrophages were cultured with MAP for 24 h.

3.5. MAP enhanced iNOS mRNA level and effect on nitric oxide production

The effect of MAP on mice peritoneal macrophages was examined since macrophages were known to be one of the target cells of polysaccharides. The stimulation of murine macrophages by LPS resulted in the expression of an inducible NO synthase (iNOS), which catalyzed the production of large amounts NO from L-arginine and molecular oxygen. As shown in Fig. 8, 30 μg/ml of MAP enhanced the level of iNOS mRNA; however, its induction was less than that of LPS at 24 h. The effect of MAP on the production of NO in peritoneal macrophages was also

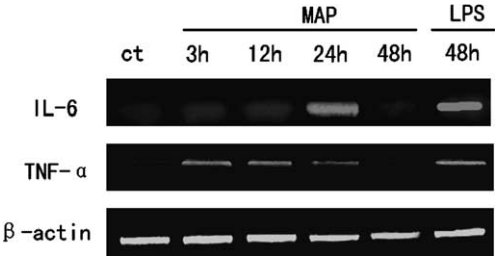

Fig. 7. RT-PCR analysis of cytokines mRNA expression in mice peritoneal macrophages stimulated by MAP. Macrophages (5×10^5cells/ml) were incubated with MAP at the concentration of 30 μg/ml up to 48 h. Total RNA was isolated at various times and was subject to RT-PCR. RT-PCR products were analyzed on 2% agarose gel electrophoresis. Product treated with LPS (5 μg/ml) was used as positive control, and products from untreated macrophages was used as control (ct).

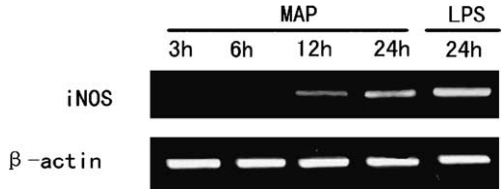

Fig. 8. Time-dependence expression of iNOS mRNA in mice peritoneal macrophages activated by MAP. Macrophages (5×10^5cells/ml) were incubated in 24-well plates with or without MAP at the concentration of 30 μg/ml up to 24 h. Total RNA was isolated at various times and level of mRNA expression of iNOS was evaluated by RT-PCR. RT-PCR products were analyzed on 2% agarose gel electrophoresis. Product treated with LPS (5 μg/ml) was used as a positive control.

investigated. Treatment of peritoneal macrophages with MAP resulted in an enhanced production of NO slightly (data not shown), which was not statistical significant.

4. Discussion

Previously, Qin, Huang, and Xu (2002c) researched the immunomodulating activities of MAP, a polysaccharide isolated from the mucus of the loach, *Misgurnus anguillicudatus*, and indicated that MAP has immunomodulating functions in animals and can enhance the immune system. In order to ensure target immune cells activated by MAP, in the present study, we investigated the immunomodulatory activity of MAP at the cellular level. Firstly, we investigate proliferation effecting of MAP on mice spleen lymphocytes and peritoneal macrophages populations. Our results demonstrate that MAP has typical immunostimulatory activity characteristic, in that it selectively activates T cells and macrophages, but not B cells. In additional, its immunomodulating activity exerts greatest on the optimal dose of 30 μg/ml, in the present study. At this concentration, MAP promoted farthest proliferation of spleen lymphocyte or macrophages.

The cell-type selectivity of MAP appears to be similar to that of lentinan, β(1→3)-glucan isolated from *Lentinus edodes*, is a stimulator of T cells and macrophages, but does not accelerate B cell antibody formation (Boehmer, 1994). Another β(1→3)-glucan schizophyllan, isolated from *Schizophyllium commune* appears to be similar to MAP in terms of its biological activity. Schizophyllan restores and enhances cellular immunity in the tumor-bearing host by functioning as a T cell adjuvant and a macrophage activator (Suzuki, Kikuchi, Takatsuki, & Hamuro, 1994). Although MAP and lentinan or schizophyllan are similar in their mode of action, their chemical compositions are different since MAP is composed of galactose, fucose and mannose (Qin et al., 2002b).

In the present study, MAP exerts the mitogenic activity and IL-2, IL-4 secretion-enhancing activity on normal thymus-dependent lymphocyte (T cells). These results suggest that the mitogenic activity and interleukin production-enhancing activity of MAP might be exerted via membrane receptor on T cell surface. Since MAP cannot penetrate cells due to its large molecular mass, this selectivity may be caused by the surface binding of this molecule to receptors specifically expressed on T cells and macrophages, but not on B cells. A further investigation of the membrane receptor of MAP should shed light on its selectivity for T cells and macrophages.

The features of the immune response triggered against a particular pathogen depend on the pattern of cytokines expressed by Th cells. Two major patterns of cytokines expressed have been recognized in murine and human T cell clones. Th1 clones produce interferon γ (IFN-γ), interleukin 2(IL-2) and tumour necrosis factor α (TNF-α), and induce a basically cell mediated immune response. Th2 clones secrete IL-4, IL-5, IL-6 and IL-10, promoting basically humoral respones (Seder & Paul, 1994).

Time-dependence analysis showed some differences action of MAP on T cells among four kinds of cytokines. IL-2 and IFN-γ responded rapidly to MAP, whereas IL-4 and IL-6 were affected after a few hours treatment with MAP, and the increased expressions IL-2 was maintained thereafter. The four cytokines are related with each other in that they are coordinately released from activated T cells, and that IL-2 can induce IL-4 or IL-6. INF-γ suppresses proliferation and secreting cytokines of Th2 cells (Gong, 1998). The expression pattern of IL-6 and IL-4 mRNA might result from the suppressive activity of IFN-γ on the expression of Th2-related cytokines.

On the other hand, IL-6 is also considered as a major immune and inflammatory mediator (Song et al., 2002). One of foremost functions of IL-2 is that it can induce proliferation of T lymphocyte (from period G_0 into period S). T cells activated can secret IL-2, which acts on T lymphocyte, induces proliferation and differentiation itself, and exerts its functions. IL-4 operates key action on viability, growth and differentiation of Th2 cells, and participates in coadjusting between Th1 and Th2 cells. Nevertheless, it suppresses immune response mediated by Th1 cells (Boehmer, 1994; Kelso, 1995; Nossal, 1994). Thereby, INF-γ mRNA expression decreased along with IL-4 suppression activity on Th1 cells.

Accordingly, this suggested that Th1 cells, which secret Il-2 and INF-γ cytokines, were primary cellular targets directly affected by MAP on T lymphocyte. Whereupon, secondary response of Th2 cells related with IL-4 and IL-6 mRNA expression were followed. Therefore, it was suggested that the interleukin secretion-enhancing activity of T cells by MAP might partially contribute to the immunomodulating functions of polysaccharide from mucus of loach. As demonstrated Fig. 4, MAP increased slightly polyclonal IgM antibody production of B cell. This might have resulted from stimulation of cytokines secreted from T cells activated by MAP.

Activated macrophages are considered to be one of the important components of the host defense against tumor

growth (Fidler & Kleinerman, 1993). The activation process includes the generation of cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-12 (IL-12), and they are directly involved in the macrophage-mediated tumor cell killing. The expression of IL-6, having a growth regulatory effect, has been implicated as one of the cytostatic/cytocidal factors in the antitumor action of activated macrophages (Hamilton & Adams, 1987; Paulnock, 1992). MAP can induce macrophages secreting TNF-α and IL-6, and time-dependence showed that expression of TNF-α mRNA was augmented early in MAP-activated macrophages. IL-6 mRNA inductions by MAP reached the maximum after 24 h of treatment. As demonstrated in Fig. 7, it has been known that activated macrophages can kill tumor cells and TNF-α is responsible for this killing. These two cytokines are related with each other in that they are coordinately released from activated macrophages. TNF-α can accelerate macrophages secreting IL-6, but IL-6 suppresses TNF-α production by macrophages. Because the mRNA of TNF-α was rapidly enhanced after 3 h treatment, MAP might be considered to be a strong stimulator of TNF-α rather than IL-6.But whether MAP directly triggered IL-6 production need await further study.

The result showed that the mRNA level of the inducible enzyme nitric oxide synthase (iNOS) was strongly by MAP at 24 h. Nitric oxide is a short-lived radical that is formed by iNOS, and the iNOS is considered to be a central molecule in the regulation of the immune response to tumors. TNF-α, IL-1β and bacterial LPS can induce the expression of iNOS in a wide variety of tissues, organs and in some tumor cell lines. The strong stimulator for the induction of iNOS is LPS, and the induction of iNOS by other stimuli also leads to organ destruction in inflammatory region. iNOS also plays a key role in host defense against infectious agents, including viruses (Song et al., 2002). In the present study, we investigated the effect of MAP on the activation of macrophages by examining the expression levels and the production of macrophage induced cytokines and NO. The results showed that the mRNA level of iNOS was strongly induced by MAP at 24 h. MAP also induced NO production at 48 h by enhancing iNOS expression primarily. However, MAP-treated cells produced less amounts of NO than LPS (data not shown). It might mean that MAP reduced cytotoxicity towards normal or immune cells.

In conclusion, the biological functions of MAP result from it selectively activating T cells and macrophages and stimulating secretion of some cytokines.

Acknowledgements

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References

Boehmer, H. V. (1994). Positive selection of lymphocytes. *Cell*, *76*, 219–228.

Borchers, A. T., Stern, J. S., Hackman, R. M., Keen, C. L., & Gershwin, M. E. (1999). Mushrooms, tumors, and immunity. *Proceedings of the Society for Experimental Biology and Medicine*, *221*(4), 281–293.

Ding, A. H., Nathan, C. F., & Stuehr, D. J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *Journal of Immunology*, *141*, 2407–2412.

Fidler, I. J., & Kleinerman, E. S. (1993). Therapy of cancer metastasis by systemic activation of macrophages: From bench to the clinic. *Research in Immunology*, *144*, 274–276.

Gong, F. L. (1998). *Immunology Basics: Lymphocyte* (1st ed.). Wuhan: Hubei Science and Technology Pub.

Goto-Nance, R., & Watanabe, Y. (1995). Characterization of lectins from the skin mucus of the loach, *Misgurnus anguillicaudatus*. *Fish Sciences*, *61*, 137–140.

Hamilton, T. A., & Adams, D. O. (1987). Molecular mechanisms of signal transduction in macrophage activation. *Immunology Today*, *8*, 151–158.

Han, S. B., Kim, Y. H., Lee, C. W., Park, S. M., Lee, H. Y., Ahn, K. S., Kim, I. K., & Kim, H. M. (1998). Characteristic immunostimulation by angelan isolated from *Angelica gigas* Nakai. *Immunopharmacology*, *40*(1), 39–48.

Han, S. B., Oh, G. T., Yun, Y. P., Min, B. K., Hyun, B. H., & Kim, H. M. (1996). Rapid determination of in vivo and in vitro antibody responses by suspension hemolytic assay. *Journal of Pharmacological and Toxicological Methods*, *36*(1), 33–40.

Han, S. B., Park, S. H., Lee, C. W., Lee, S. H., Kim, H. C., Kim, Y. S., Lee, H. S., & Kim, H. M. (2001). Polysaccharide isolated from the radix of *Platycodon grandiflorum* selectively activates B cells and macrophages but not T cells. *International Immunopharmacology*, *1*(11), 1969–1978.

Kelso, A. (1995). T_H1 and T_H2 subsets: Paradigms lost? *Immunology Today*, *16*, 374–379.

Kim, H. M., Han, S. B., Oh, G. T., Kim, Y. H., Hong, D. H., Hong, N. D., Hong, N. D., & Yoo, I. D. (1996a). Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. *International Journal of Immunopharmacology*, *18*(5), 295–303.

Kim, H. M., Oh, G. T., Hong, D. H., Hyun, B. H., Cha, Y. N., Yoo, B. S., & Han, S. B. (1996b). Facilitation of apoptosis by autologous serum and related immunosuppression in the splenocyte culture. *Immunopharmacology*, *34*, 39–50.

Klimetzek, V., & Remold, H. G. (1980). The murine bone marrow macrophages, a sensitive indicator cell for murine migration inhibitory factor and a new method for their harvest. *Cellular Immunology*, *53*, 257–266.

Liu, F., Ooi, V. E. C., & Fung, M. C. (1999). Analysis of immunomodulating cytokine mRNA in the mouse induced by mushroom polysaccharides. *Life Sciences*, *64*(12), 1005–1011.

Mariko, K., Yoichiro, H., Toshhisa, S., & Makio, A. (1994). Characterization of a deaminated neuraminic acid-containing glyciprotein from the skin mucus of the loach, *Misgurnus anguillicaudatus*. *Journal of Biological Chemistry*, *269*(51), 32138–32143.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*, 55–63.

Nossal, G. J. V. (1994). Negtive selection of lymphocytes. *Cell*, *76*, 229–239.

Okutumi, T., Nakajima, Y., Sakakibara, F., Kawauchi, H., & Yamazaki, M. (1987). Induction of a cytotoxin from murine bone narrow by an animal lectin. *Cancer Research*, *47*, 47–50.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, *7*(7), 715–729.

Park, C. B., Lee, J. H., Park, I. Y., Kim, M. S., & Kim, S. C. (1997). A novel antimicrobial peptide from the loach *Misgurnus angullicaudatus*. *FEBS Letters*, *411*, 173–178.

Paulnock, D. M. (1992). Macrophage activation by T cells. *Current Opinion in Immunology*, *4*(2–3), 344–349.

Qin, C. G., Huang, K. X., & Xu, H. B. (2002a). Protective effect of polysaccharides from the loach on the in vitro and in vivo peroxidative damage of hepatocyte. *Journal of Nutritional Biochemistry*, *13*(10), 592–597.

Qin, C. G., Huang, K. X., & Xu, H. B. (2002b). Isolation and characterization of a novel polysaccharide from the mucus of the loach, *Misgurnus anguillicaudatus*. *Carbohydrate Polymers*, *49*(3), 367–371.

Qin, C. G., Huang, K. X., & Xu, H. B. (2002c). Investigation on immunomodulating activities of polysaccharide from loach mucus. *Chinese Pharmaceutical Journal*, *37*(8), 588–591.

Seder, R. A., & Paul, W. E. (1994). Acquisition of lymphokine-producing phepotype by CD^+ T cells. *Annual Review of Immunology*, *12*, 635–673.

Song, J. Y., Han, S. K., Son, E. H., Pyo, S. N., Yun, Y. S., & Yi, S. Y. (2002). Induction of secretory and tumoricidal activities in peritoneal macrophages by ginsan. *International Immunopharmcoology*, *2*(7), 857–865.

Sun, W. M., & Wang, H. Q. (1999). *Research methods of cytokines: Separating B lymphocyte by the nylon-wool methods* (1st ed.). Beijing: People's Sanitation Pub. Inc.

Suzuki, M., Kikuchi, T., Takatsuki, F., & Hamuro, J. (1994). Curative effects of combination therapy with lentinan and interleukin-2 against established murine tumors, and the role of CD8-positive T cells. *Cancer Immunology, Immunotherapy*, *38*(1), 1–8.