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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,