Effect of *Misgurnus anguillicaudatus* polysaccharide on immune responses of splenocytes in mice

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ABSTRACT

AIM: To investigate the effect of *Misgurnus anguillicaudatus* polysaccharides (MAP) on immune responses of splenocytes in mice. **METHODS**: T lymphocyte proliferation (TLP) was measured by [^{3}H] thymidine incorporation assay. Cytotoxic T lymphocyte (CTL) cytotoxicity and natural killer (NK) activity were determined by release of radioactive chromium [^{51}Cr] from pre-labeled target cells. **RESULTS**: MAP 5 and 10 mg·kg^{-1}·d^{-1}, ip for 7 d, could increase TLP, enhance the cytotoxicity of CTL and NK cells, and antagonize the effect of concanavalin A (ConA) on TLP suppressed by cyclophosphamide (CP). Inhibitory rates of CTL cytotoxicity were decreased from 51.4 % in CP control mice to 18.2 % and 35.1 % in MAP-treated CP mice, respecitvely. Furthermore, administration of MAP 10 and 20 mg·kg^{-1}·d^{-1}, ip for 7 d, restored the reduced NK cell cytotoxicity caused by CP administration in mice. **CONCLUSION**: MAP has protective effect on augmenting T-cell-mediated immunity and NK activity in normal and CP-treated mice.

INTRODUCTION

It is widely recognized that phagocytosis plays an outstanding role in the defense of human and animals from numerous infectious and non-infectious factors. Since phagocytes act as regulatory and effective cells in the immune system, the enhancement of phagocyte function is expected to be applicable for therapy of microbial infection and cancer. Cell-mediated immunity plays an important role in host resistance against tumor development and infection by microbial organisms[1,2]. Particularly, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells can directly kill certain tumor cells[3,4]. Depression of their function will decline the antitumor ability of the host.

Polysaccharides from plant and fungi are well known to possess an immunomodulating activity that primarily implies the non-antigen dependent stimulation of the function and efficiency of granulocytes and macrophages. Despite the numerous polysaccharides with the reported immuostimulating function[5], it is of great interest to search for new sources. Immunoactive polysaccharides have been also isolated from the cell culture of some plants[6]. But the reports on the immunoactive polysaccharides from fish have rarely been seen.

The loach (*Misgurnus anguillicaudatus*) has long been employed as traditional Chinese medicine in folk remedies for the treatment of hepatitis, osteomyeitis, carbuncle, inflammation, and cancer, as well as for the restoration of health in debilities caused by various pathogenes and aging. *Misgurnus anguillicaudatus* polysaccharide (MAP) was extracted from the mucus of the loach and found to have effects on removal of reactive oxygen species and protection of DNA chains[7]. The present study was carried to examine whether MAP could enhance the cytotoxicity of CTL and NK cells. The protective effect of MAP on the suppressed immune function of T, CTL, and NK cells by cyclophosphamide was also investigated.

MATERIALS AND METHODS

Materials   MAP was isolated and purified by our research group and the homogeneity and molecular weight of MAP were determined by gel permeation chromatography (GPC) with a Waters HPLC apparatus. The chromatography gave a single sharp peak and showed that the average M_r of MAP was 130 300. The analysis by gas chromatography indicated that MAP mainly composed

of *D*-galactose, *L*-fucose, and *D*-mannose[7]. Drugs such as cyclophosphamide (CP, № 12 Pharmaceutical Factory of Shanghai) and Levamisole (LMS, № 5 Pharmaceutical Factory of Shanghai) were dissolved in sterile 0.9 % NaCl solution, respectively. The chemical reagents were commercially available.

Animals Female Kunming mice, SPF grade, 5–6 weeks old at the beginning of the experiments, weighing 20 g ± 2 g, were provided by Hubei province Medical Research Center, Wuhan, China (Certificate № 19082). Female C57BL/6 mice, 7–9 weeks old, body weight 20 g ± 2 g, were purchased from Animal Center of Academy of Military Medical Science, Beijing, China (Certificate № 19064).

Culture medium Culture Medium RPMI-1640 (Gibco, New York, USA), containing 15 % calf bovine serum, 2-mercaptoethanol 50 μmol/L, benzylpenicillin 100 kU/L, and streptomycin 100 mg/L was kept in 5 % CO_2 at 37 ℃ and used as culture medium.

Preparation of spleen cells Spleen cells prepared from groups of mice were collected aseptically, pooled on ice, and squeezed in nylon sack in Hank's solution. Then, the cells were suspended in culture medium and adjusted to the stated cell concentrations, cell viability was over 90 %. Mastocytoma P815 cell (provided by Beijing Medical University) or leukemia Yac-1 cells (supplied by Beijing Institute of Radiology) grown in suspension cultures were harvested with [^{51}Cr] (specific activity 925 GBq/g, Beijing Institute of Nuclear Energy). Half mL of the cell suspension in a cell density of 4×10^8/L were incubated with 3.7–7.4 MBq of radioactive [^{51}Cr] in 5 % CO_2 for 1 h at 37 ℃. Then, the cells were suspended and washed five times with culture medium[8]. The cell suspensions were then adjusted to 2×10^7/L.

T lymphocyte proliferation test T lymphocyte proliferation was measured by [^{3}H] thymidine (radio-concentration 99.9 MBq/L, Shanghai Institute of Nuclear Energy) incorporation assay[9].

Cytotoxic T lymphocyte cytotoxicity[10] Female C57BL mice were sensitized by ip injection of 5×10^6 mastocytoma P815 cells in ascitic form. Spleen cells were harvested 12–15 d later. Spleen cell suspensions containing 2×10^6 cells were mixed with equal volumes of pre-labeled target cells containing 2×10^4 cells. Two mL of the reaction mixtures were placed in round bottom tubes and the cells incubated in 5 % CO_2 at 37 ℃ for 4.5 h. At end of incubation,

1 mL of supernatant was removed and the radioactive was measured in a gamma counter.

The cytotoxic effect of CTL was calculated from the following formula:

CTL cytotoxicity (%) = (A/B) × 100 %

A = [^{51}Cr] release in the presence of immune lymphocytes – [^{51}Cr] release in the presence of normal lymphocytes

B = Maximum release – [^{51}Cr] release in the presence of normal lymphocytes

The maximum release was determined by the addition of Triton X-100 instead of effect cells.

Assay for NK activity Spleen cells 4×10^6/L from female Kunming mice were mixed with equal volumes of [^{51}Cr] labeled Yac-1 cells 2×10^7/L. Two mL of the reaction mixtures were added in each of the triplicate round bottom tubes. After incubation (at 37 ℃ for 4.5 h), 1 mL supernatant was measured in a gamma counter[11]. The percentage of [^{51}Cr] release was calculated from the counts in mean value of triplicate tubes according to the following formula:

NK activity (%) =

$$\frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100 \%$$

Spontaneous release was the release (cells/mL) from the target cells incubated in the culture medium without effect cells, maximum release was determined by the release from the target cells incubated in 2 % Triton X-100. Spontaneous release was below 15 %, and maximum release was over 85 % of the release from the target cells.

Statistical analysis The data shown were expressed as $x \pm s$. Significance of difference was calculated by Student's *t*-test.

RESULTS

Influence of MAP on immune response of T, CTL, and NK cells in normal mice The effect of MAP on splenic T lymphocyte proliferation induced by concanavalin A (Con A) were shown in Tab 1. MAP 5 or 10 mg/kg, ip, could increase the splenic T lymphocyte proliferation in normal mice.

Female C57BL mice were divided into 4 groups. The first group served as control. The other 3 groups were inoculated P815 cells 5×10^6 cells/mice on d 0. The results showed that levamisole (LMS, 10 mg/kg, ip) could increase the CTL cytotoxicity and MAP (5 mg/kg, ip) beginning on d 8 could significantly

increase the CTL cytotoxicity (Tab 2).

Tab 1. Effect of MAP (5 or 10 mg/kg, ip for 7 d) on splenic T lymphocyte proliferation in normal mice. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ vs control.

MAP/mg·kg^{-1}	[^{3}H]TdR incorporation/Bq	
	Without ConA	With ConA (10 mg/kg)
0 (Control)	60 ± 3	376 ± 41
5	56.1 ± 2.0	503 ± 34^c
10	63 ± 4	871 ± 32^c

Tab 2. Effect of MAP (10 mg/kg, ip, for 7 d) on cytotoxic function of CTL in immunized mice. $n = 8$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ vs immunized mice without drug.

Group	Supernatant/Bq	Specific cytotoxicity/%
Control	2.80 ± 0.2	–
Immunized	8.00 ± 1.7^b	31.5
Immunized + LMS	10.8 ± 1.9^b	50.2
Immunized + MAP	14.7 ± 0.9^c	69.1

MAP (5 mg/kg, ip) could increase NK cell cytotoxicity in normal mice. MAP (10 mg/kg, ip) did not increase NK cell cytotoxicity (Tab 3).

Tab 3. Effect of MAP on splenic natural killer cytotoxicity in mice. MAP was administered ip for 7 d, beginning on d 0, and splenic natural killer cytotoxicity was assayed on d 7. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ vs control.

MAP/mg·kg^{-1}	Supernatant/Bq	Cytotoxicity/%
0 (Control)	5.40 ± 0.19	13.0
5	6.40 ± 0.20^c	16.9
10	3.40 ± 0.20	10.1

Effect of MAP on the immune function of T, CTL, and NK cells in CP-induced immunosuppressed mice   The enhancement of splenic lymphocyte proliferation by MAP in CP-treated mice. CP (25 mg/kg, sc) could depress relative proliferation index (RPI) fell from 100 % to 34 % on d 7. When MAP was administered intraperitoneally 10 mg·kg^{-1}·d^{-1} × 7 d, beginning on d 0, the splenic T lymphocyte proliferation induced by ConA in CP-treated mice was significantly restored, RPI rose from 34.2 % to 103 % on d 7 (Tab 4).

Tab 4. Enhancement of splenic lymphocyte proliferation by MAP in CP-treated mice. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ vs CP alone. dRPI: relative proliferation index = Δ cpm of test mice/Δ cpm of control mice × 100 %, Δcpm = cpm with ConA – cpm without ConA.

Drug	[^{3}H]TdR incorporation/Bq		dRPI/%
	Without ConA	With ConA (10 mg/kg)	
Control	35 ± 4	126 ± 13	100
CP	36 ± 6	67 ± 9	34.2
CP + MAP	34 ± 6	127 ± 3^c	103

In our experiments, female C57BL mice were administered CP (50 mg/kg) on d 6 after P815 cells were inoculated and the CTL cytotoxicity determined on d 12. CP markedly inhibited CTL activity, treatments with MAP 5 or 10 mg·kg^{-1}·d^{-1} × 7 d, ip, induced an enhancement of the suppressed CTL cytotoxicity (Tab 5).

Tab 5. Enhancement of splenic CTL cytotoxicity by MAP in mice treated with CP. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ vs CP alone.

Group	Supernate/Bq	Specific cytotoxicity/%	Inhibitory rate/%
Immunized	12.1 ± 0.5	69.8	0
Immunized + CP	8.1 ± 0.4	33.9	51.4
Immunized + CP + MAP 5	10.8 ± 0.4^c	57.1	18.2
Immunized + CP + MAP 10	9.3 ± 0.2^c	45.3	35.1

Another aspect of cytotoxicity is natural killer activity using Yac-1 tumor cells as targets. Effect cells were spleen cells from control mice, CP-treated mice and CP + MAP treated mice. After administered with a single dose of CP (50 mg/kg, sc) on d 0, the mice were served with MAP (10 or 20 mg·kg^{-1}·d^{-1}, ip for 7 d), beginning on d 0. Splenic NK cytotoxicity was assayed on d 7. CP 50 mg/kg inhibited the NK cell activity, it was restored by MAP 10 or 20 mg·kg^{-1} (Tab 6).

DISCUSSION

The mucus coat of fish skin contains a variety of secretions from epidermal goblet cells and epithelial cells. These secretions have been implicated in many important biological functions[12]. Some vertebrate lectins were

Tab 6. Enhancement of splenic NK cell cytotoxicity by MAP in mice treated with CP. $n = 8$. $\bar{x} \pm s$. $^{b}P < 0.05$ vs CP alone.

Drug/mg·kg^{-1}	Supernatant/Bq	Cytotoxicity/%
Control	5.4 ± 0.2	13.2
CP	4.7 ± 0.2	10.3
CP + MAP 10	5.8 ± 0.2^{b}	16.4
CP + MAP 20	5.8 ± 0.3^{b}	17.2

purified from the skin mucus or egg of the loach and found to induce release of cytotoxin from fresh murine bone marrow cells or macrophages and lyse tumor cells but not normal spleen cells[13]. A novel antimicrobial peptide named misgurin, which consists of 21 amino acids, from the loach, had been isolated and identified[14]. The carbohydrate compositions, a deaminated neuraminic acid-containing glycoprotein from the skin mucus of the loach was isolated and characterized[15].

In this work, the effects of MAP on several lymphocyte functions has been investigated in normal mice as well as in immuno-suppressed mice. Initial results obtained in normal mice showed that ip treatments with MAP increased the immune functions of T, CTL, and NK cells. Further, we administered CP plus MAP to several group of mice. The results indicated that MAP was effective in restoring T, CTL, and NK cell activity which has been suppressed by CP.

Growing evidences showed that T, CTL, and NK cells played a central role in preventing or modulating tumor growth. Administration of MAP augmented the CTL and NK cell cytotoxicity of spleen cells in normal mice. Furthermore, the suppressed cytotoxicities induced by CP were restored completely or partially but significantly by MAP. These results suggested that enhancement of cytotoxicity of T, CTL, and NK cells might be one possible mechanism of the antitumor activity of MAP.

CP has been available since 1959 and subjected to extensive study as a chemotherapeutic agent of cancer. However, this cytotoxic drug exerts toxic effects on both normal and tumor tissues even at optimal doses. The lack of selective toxicity results in adverse effects, especially, the dysfunction of immune system and is the major limiting factor in the chemotherapy of cancer. The enhancement of suppressive immune function by MAP in CP-treated mice provides a means to compensate some defects in chemotherapy.

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泥鳅多糖对小鼠脾细胞免疫应答的影响

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关键词 泥鳅; 多糖类; 环磷酰胺; T 淋巴细胞;
细胞毒性 T 淋巴细胞; 自然杀伤细胞

目的: 研究泥鳅多糖对小鼠脾细胞免疫应答的影响.
方法: 分别给正常小鼠、刀豆蛋白(ConA)或左旋咪
唑免疫增强小鼠、环磷酰胺免疫抑制小鼠腹腔注射
提取的泥鳅多糖, 连续给药 7 d. 用[3]H-胸苷掺入法
测定 T 淋巴细胞的增殖. 用[51]Cr 同位素标记法测定
细胞毒 T 淋巴细胞的细胞毒性和天然杀伤细胞的活
性. **结果**: 泥鳅多糖 5 或 10 mg·kg^{-1}·d^{-1}可以提高
T 淋巴细胞的增殖, 并增强细胞毒 T 淋巴细胞的细
胞毒性和天然杀伤细胞的活性, 还能增强 ConA 解
除环磷酰胺对 T 淋巴细胞增殖抑制的能力, 抑制率
从环磷酰胺对照组的 51.4 % 分别降低到 18.2 % 和
35.1 %. 而且, 给予小鼠 10 或 20 mg·kg^{-1}·d^{-1}的
泥鳅多糖, 可以恢复环磷酰胺所致的天然杀伤细胞
活性降低. **结论**: 泥鳅多糖能增强小鼠脾细胞中 T
细胞、细胞毒 T 淋巴细胞和天然杀伤细胞的活性.