Production of macrophage activating factors by the mitogen-stimulated lymphocytes of Japanese parrotfish (*Oplegnathus fasciatus*) and the properties of this factor

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Spleen lymphocytes were prepared from Japanese parrotfish (*Oplegnathus fasciatus*) and incubated with 100 mg/ml of Concanavalin A bound to Sepharose 4B beads for 48h, and then the stimulated cell supernatants were collected. Thyoglycolate induced, allogenic, peritoneal macrophages were incubated with or without stimulated cell supernatants. Then, the phagocytic activity against inactivated yeast and peroxidase production were measured. Stimulated cell supernatants increased the phagocytic activity and the peroxidase production of allogenic peritoneal macrophages. The activities of these stimulated cell supernatants were lost completely when they were heated at 56℃ for 30 min or dialyzed against a pH 2 buffer for 24h-similar to IFN-γ. Stimulated cell supernatants did not increase phagocytic activity and peroxidase production of heterogenic mouse peritoneal macrophages.

These findings suggest that the spleen lymphocytes of Japanese parrotfish can produce a macrophage activating factor *in vitro* with Concanavalin A bound to Sepharose 4B beads, and that this factor may be IFN-γ.

Key Words：peritoneal macrophage, phagocytic activity, IFN-γ, spleen lymphocytes, Japanese parrotfish (*Oplegnathus fasciatus*), Concanavalin A

Macrophage-histiocyte series cells provide important information about how hosts defend themselves against pathogens and tumor cells.[1-3] This defence activity is thought to involve cooperation between lymphocytes and macrophages. Mammalian macrophages can be activated by *in vitro* interaction with lymphokines in stimulated cell supernatant or γ-interferon (IFN-γ).[4-6] In addition, IFN-γ is a macrophage activating factor (MAF) and it displays antiviral activity in mammalian immunosystems.[7] Many infectious diseases caused by pathogenic viruses, such as viral nervous necrosis of Japanese parrotfish,[8] striped jack[9] and iridovirus infection of red sea bream[10] are reported in aquaculture fields. However, little is known about the immune defence response to pathogenic bacteria and virus in fish.[11]

In this paper, we report that the spleen lymphocytes of cultured Japanese parrotfish (*Oplegnathus fasciatus*) can produce a macrophage activating factor (MAF) following *in vitro* incubation with Concanavalin A (Con A) bound to Sepharose 4B beads, and that this MAF activity is similar to that of IFN-γ.

Materials and methods

Materials

Ten month old cultured Japanese parrotfish (*Oplegnathus fasciatus*), with a mean body weight of 80±10 g were obtained from the Nagasaki Municipal Fisheries Center (Nagasaki, Japan). Fish were sacrificed by bleeding them from their gill vein under anesthesia with 100 ppm of MS-222.

Mice

Ten to 11-week-old specific pathogen-free C57BL/6 male mice were obtained from the Japan Clear Inco. (Tokyo, Japan). Mice were sacrificed by bleeding them from their axillary vein under ethyl ether anesthesia.

Preparation and purification of macrophages

Thioglycolate-elicited, peritoneal macrophages (Pmϕ) were obtained 5 days after an intraperitoneal injection (5 ml for fish, 1 ml for mice) of thioglycolate using a peritoneal lavage.[4] Briefly, the peritoneal cavity was

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washed several times with sterilized saline (containing 5 U/ml heparin) so that a total of 20 ml fluid was used per fish or 15 ml fluid per mouse. The peritoneal lavage suspension was washed and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, penicillin G, and streptomycin (named CRPMI 1640). The viability of nucleated cells as measured by trypan-blue dye exclusion, was >95%. The total number of cells collected was determined with a hemocytometer (using 2% acetic acid as diluent). The Pmϕ content in the CRPMI 1640 cell suspension was determined using a morphological stain (May-Giemsa stain). Then, $3x10^6$ Pmϕ suspensions were plated into the wells of Microtest plates (200 μl/well) or on petri dishes (Falcon 3001 Plastics, Oxnerd, CA) with a sterilized cover slip for 60 min in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were removed by washing twice with RPMI 1640. More than 90% of the adherent cells from the peritoneal cavity were Pmϕs, judging by their positive staining for nonspecific esterase.

Preparation of Con A-stimulated cell supernatants from splenocytes

The preparation of Con A-stimulated cell supernatants from splenocytes was described in detail previously.[4] Briefly, the spleens from normal mice and fish were aseptically collected and minced in CRPMI 1640. The splenocyte suspension was then washed with medium and passed through a 27-gauge needle for preparation of single cells. The viability of the mononuclear cells was >95%, as determined by tripan blue exclusion. Splenocytes were adjusted to 2 x 10^6 cells/ml with CRPMI 1640. Con A bound to Sepharose 4B beads (Sigma Chemical Co. ST. Louis, MO) was added to the splenocyte suspension to make up a final concentration of 100 μg bound Con A/ml. Then the cells were put into 50 μl tissue culture flasks (Falcon 3013 Plastics, Oxnard, CA) and incubated at 37℃ for mice or at 25℃ for fish. After 48 h, the suspensions were centrifuged at 3,000 rpm for 20 min to remove the cells and Sepharose 4B beads. The supernatants were filtered through a sterile 0.45 μm Millipore membrane and then stored at -70℃.

In vitro activation of Pmϕ

Pmϕs were plated and the resulting monolayers were washed 60 min later. Then, they were incubated for 4 h with and without Con A-stimulated cell supernatants. The Pmϕ monolayers were then washed and assayed for phagocytosis and peroxidase production.

Phagocytosis assay

The association of Pmϕ with inactivated yeast cells (*Saccharomyces cerevisiae*) was studied by a modification of the method of Schmid and Brune.[12] After *in vitro* activation, Pmϕ monolayers were washed twice with RPMI 1640. Then 2 ml of fresh CRPMI 1640 containing 2 x 10^6 yeast cells was introduced into each dish and they were incubated for 30-120 min. The cells adhering to the cover slip, consisting mainly of macrophages, were then rinsed twice with 0.1 M phosphate buffer (pH 7.2) and stained with May-Giemsa. The number of cells associated with one or more yeast cells, among 2 x 10^2 cells, was counted.

Peroxidase production assay

Peroxidase production was examined as follows. After the *in vitro* activation, the Pmϕ monolayers on the microtest plates were washed three times with phosphate buffered saline. Then Pmϕ cell wells were broken by two rounds of freezing and thawing. Finally, 100 μl of ABTS (0.54 g of 2,2-azinobis [3-ethylbenzthiazoline]-6 sulfonic acid and 21.0 g of citric acid monohydrate in 1l of water, pH 4.2, with final concentration of 0.03% H_2O_2) was added to each well. The optical density was measured 30 min later using a Titertec Multiskan plate reader at a wavelength of 405 nm.

Heat (56℃) treatment of the Con A-stimulated cell supernatants

The heat stability of the Con A-stimulated cell supernatants was examined as follows. Samples of the supernatant were placed in individual tubes (Falcon 2097 Plastics, Oxnard, CA) and heated for 30 min at 56℃ in a water bath. Then the tubes were immersed in an ice bath and their contents were used in the *in vitro* activation for the Pmϕ.

Acid treatment (pH 2) of the Con A-stimulated cell supernatants

Acid treatment (pH 2) of the Con A stimulated cell supernatants was examined as follows. Samples of the supernatant were dialyzed in cellulose tubing against 0.1 M glycine-hydrochloric acid buffer (pH 2) for 24 h. The acid-treated supernatant was readjusted to pH 7 by dialyzing it against 0.1 M phosphate buffer (pH 7) for 24 h. Then, the supernatants were filtered through a sterile 0.45 μm Millipore membrane and were used in the *in vitro* activation of Pmϕ.

Statistical analysis

The statistical significance of the differences between test groups was analyzed by the Student's two-tailed *t*-test.

Results

Enhancement of fish Pmφ phagocytosis and peroxidase activity by Con A-stimulated cell supernatants

We added yeast suspension to Pmφ monolayers, and phagocytosis assays were terminated 30, 60, and 120 min later. As shown in Fig.1, during the 60 min. phagocytosis assay, Pmφs that were treated with Con A-stimulated cell supernatants were reproducibly and significantly more phagocytic than the Pmφs treated with NR supernatants (after the 4 h incubation). Therefore, all subsequent assays were terminated after 60 min of Pmφ incubation with yeast. We also tested different dilution factors to determine the optimum dose of stimulated cell supernatants for the enhancement of Pmφ phagocytosis (Fig. 2) and peroxdase activity (Fig. 3). The phagocytic activity of Pmφs treated, *in vitro*, with a 1/100 dilution of stimulated cell supernatant (for 4 h in CRPMI 1640) and the peroxidase activity of Pmφs treated, *in vitro*, with a 1/150 dilution of stimulated cell supernatant were both higher than those treated with NR supernatant or the medium only.

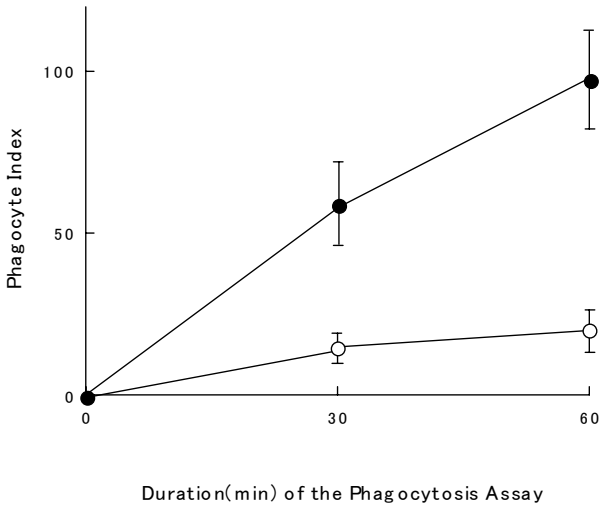

Fig.1 Phagocytosis of inactivated yeast by Pmφ treated with Con A-stimulated supernatants. Pmφ monolayers were incubated for 4 h in FCRPM 1640 with Con A-stimulated supernatants which were diluted 100 times with medium (●) or normal supernatants (NR sup.: ○). Then, the Pmφ were washed and subjected to a phagocytosis assay. Assays were terminated at the indicated times. Each point indicates the mean ± SD for triplicate cultures.

$$\text{Phagocyte index} = \frac{\text{No. of yeast}}{\text{No. of Pm}\phi} \times \frac{\text{No. of Pm}\phi \text{ phagocyted yeast}}{\text{No. of Pm}\phi} \times 100$$

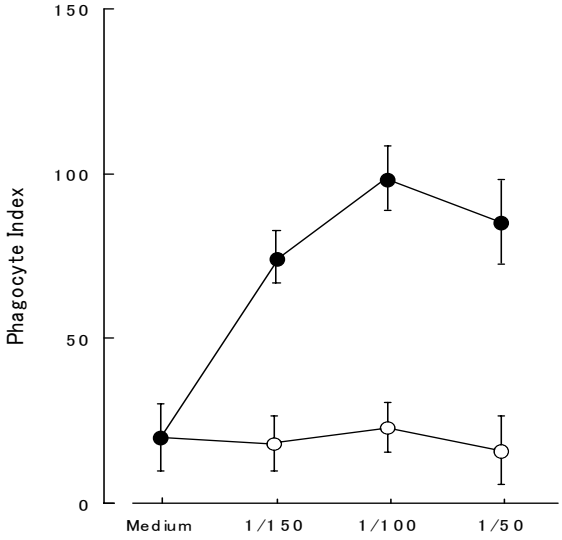

Fig.2 Augmentation of phagocytic capacity of Pmφ by Con A-stimulated supernatants Pmφ. monolayers were incubated for 4 h with the indicated dilutions of Con A-stimulated supernatants (●) or normal supernatants (NR sup.: ○). Phagocytosis assay was terminated after 60 min. Each point indicates the mean ± SD for triplicate cultures in three independent experiments. Phagocyte index was calculated as Fig. 1, respectively.

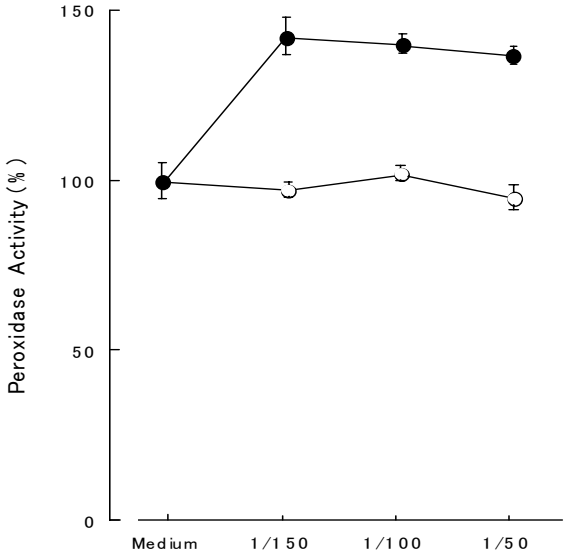

Fig.3 Augmentation of peroxidase activity of Pmφ by Con A-stimulated supernatants Pmφ. monolayers were incubated for 4 h with the indicated dilutions of Con A-stimulated supernatants (●) or normal supernatants (NR sup.:○), then peroxidase activity was measured. Each point indicates the mean ± SD for triplicate cultures in three independent experiments. Peroxidase activity (%) was calculated against optical density of medium alone.

Heat and acid treatment of Con A-stimulated cell supernatants

We examined the effects of heat and acid treatment on the macrophage activating factor (MAF) activity of stimulated cell supernatants. Incubation with heat or acid treated Con A-stimulated cell supernatants did not enhance Pmϕ phagocytosis (Fig.4) or peroxidase activity (Fig.5). Accordingly, the MAF activity of Con A-stimulated cell supernatants was lost completely when they were heated for 30 min at 56℃ or dialyzed against pH 2 buffer for 24 h.

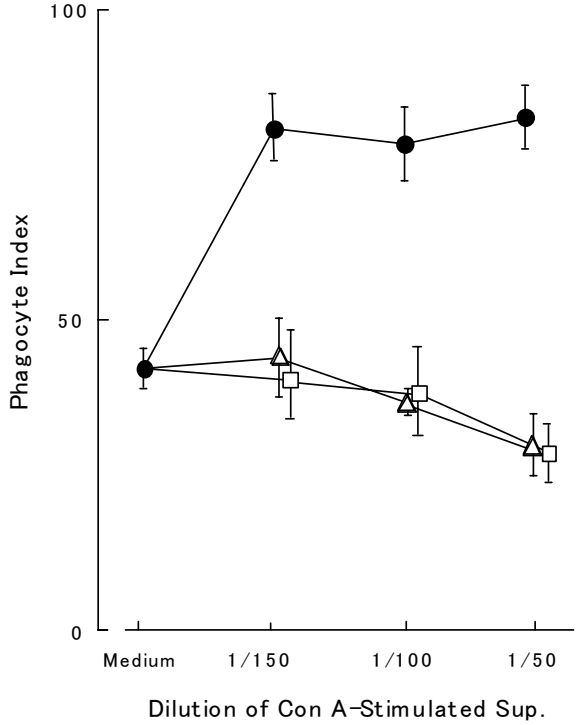

Fig.4 Effects of acid (pH 2) or heat (56℃) treatment of Con A-stimulated supernatants on phagocytic activity of Pmϕ. Acid treatment of Con A-stimulated supernatants was carried out by dialysis against 0.1 M glycine-hydrochloric acid buffer (pH 2) for 24 h. Heat treatment of Con A-stimulated supernatants was carried out by heating at 56℃ for 30 min. Pmϕ monolayers were incubated for 4h with the indicated dilutions of non-(●), acid-(△) or heat-(□) treated Con A-stimulated supernatants. The phagocytic activity was assayed in the same way as described in Fig. 2. Each point indicates the mean ± SD for triplicate cultures in two independent experiments. Phagocyte index was calculated as Fig.1, respectively.

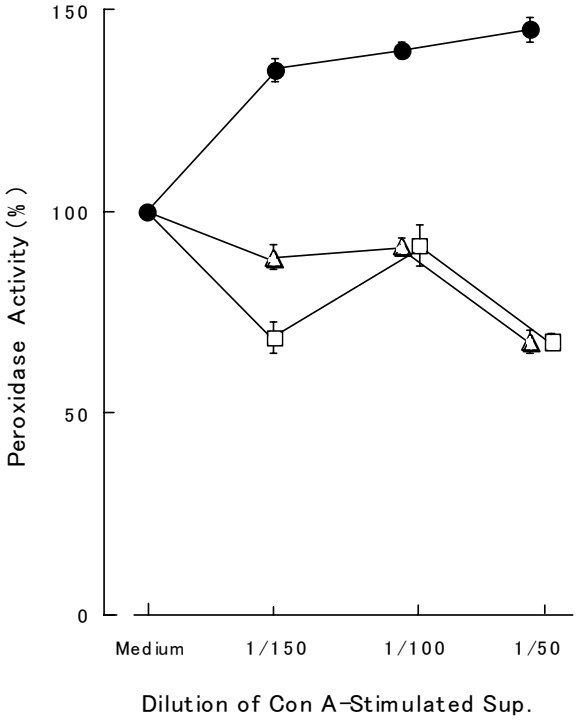

Fig.5 Effects of acid (pH 2) or heat (56℃) treatment of Con A-stimulated supernatants on peroxidase activity of Pmϕ. Pmϕ monolayers were incubated for 4 h with the indicated dilutions of non-(●), acid-(△) or heat-(□) treated Con A-stimulated supernatants, and then peroxidase activity was measured. Each point indicates the mean ± SD for triplicate cultures in two independent experiments. Peroxidase activity (%) was calculated against optical density of medium alone.

Effect of heterogeneic-stimulated cell supernatants on mouse Pmϕ phagocytosis and peroxidase activity

Next we examined whether *in vitro* treatment of stimulated cell supernatants from fish for heterogenic-stimulated cell supernatants could activate mouse Pmϕ. As shown in Fig.6, mouse Pmϕ were incubated with various dilutions of syngeneic- (from mouse) or heterogenic- (from fish) stimulated cell supernatants for a prescribed time. Phagocytosis of mouse Pmϕ increased by following incubation with syngeneic-stimulated cell supernatant (1/150 and 1/100) from a mouse. However, there was no increase observed with a higher concentration (1/50) of syngeneic-stimulated cell supernatant. Phagocytosis of mouse Pmϕ incubated in heterogenic-stimulated cell supernatant did not increase. Peroxidase activity of mouse Pmϕ increased following incubation with syngeneic-stimulated cell supernatant (1/150 and 1/50) from a mouse (Fig.7). However, the peroxidase activity of mouse Pmϕ incubated in heterogenic-stimulated cell supernatant did not increase.

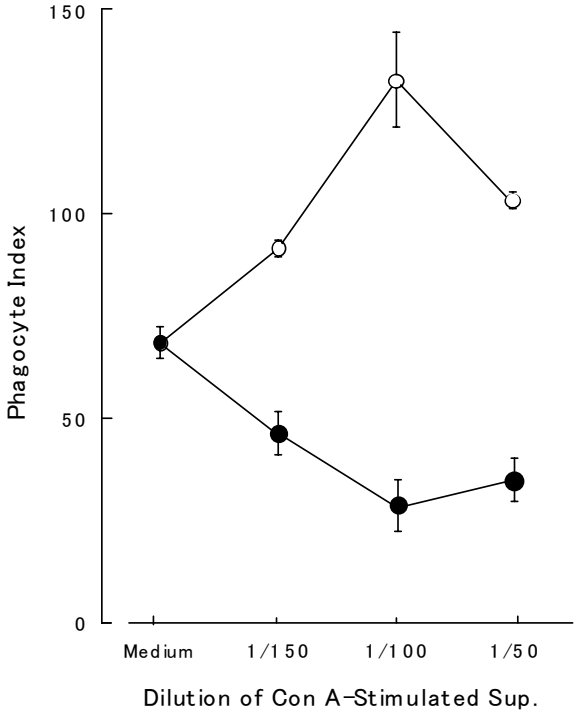

Fig.6 Effect of a different splenocyte source of Con A-stimulated supernatants on the phagocytic activity of mouse Pmϕ. Mouse Pmϕ were incubated for 4h with the indicated dilutions of Con A-stimulated syngeneic (mouse: ○) or heterogenic (Japanese parrotfish: ●) splenocytes supernatants for 4 h. The phagocytosis assay was terminated after 120 min. Each point indicates the mean ± SD for triplicate cultures in two independent experiments. Phagocyte index was calculated as Fig. 1, respectively.

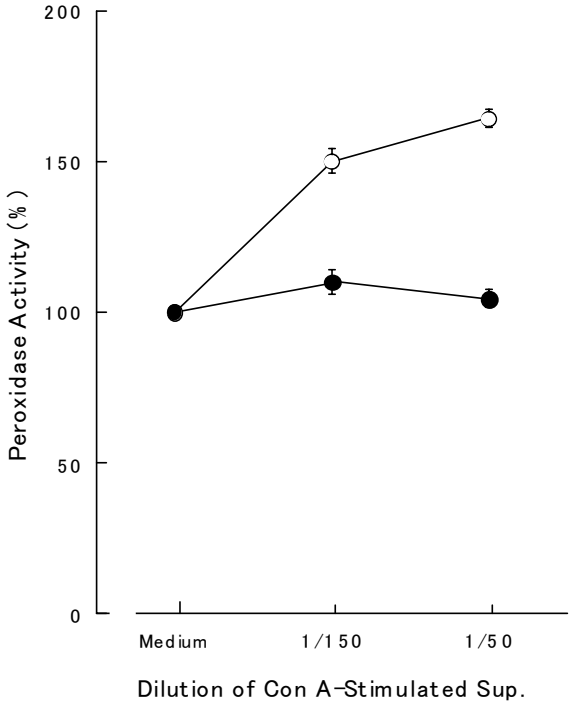

Fig.7 Effect of a different splenocyte source of Con A-stimulated supernatants on peroxidase activity of mouse Pmϕ. Mouse Pmϕ were incubated for 4 h with the indicated dilutions of Con A-stimulated syngeneic (mouse: ○) or heterogenic (Japanese parrotfish: ●) splenocytes supernatants for 4 h and then peroxidase activity was measured. Each point indicates the mean ± SD for triplicate cultures in two independent experiments. Peroxidase activity (%) was calculated against optical density of medium alone.

Accordingly, mouse Pmϕ needed syngeneic-stimulated cell supernatant from a mouse.

Discussion

In the present study, spleen lymphocytes from Japanese parrotfish produced MAF in a culture with Con A that activated phagocytosis and peroxidase production of allogenic Pmϕ. Mammalian lymphocytes can produce lymphokines including IFN-γ by contact with specific antigens or by stimulation from mitogens such as Con A or phitohemaguritinine. Also, lymphokines like IFN-γ can activate macrophage phagocytosis and tumor cytotoxicity.[4,13] On the other hand, Hardie *et al*. (1994)[14] have shown that salmon macrophages were activated by phorbol myristate acetate stimulated leucocyte supernatant. The stimulated cell supernatants from Japanese parrotfish in this experiment may contain IFN-γ.

The MAF activity of the stimulated cell supernatants from Japanese parrotfish was lost completely after heat treatment (at 56℃ for 30 min) and after acid treatment (pH 2 for 24 h). Also, the stimulated cell supernatants from fish did not activate heterogenic mouse Pmϕ. However, the Pmϕ monolayer from Japanese parrotfish incubated with stimulated, allogenic cell supernatant showed significantly greater ability to phagocytize inactivated yeast and to produce peroxidase than the same Pmϕ incubated with medium alone. Mammalian IFN-γ loses activity after heat treatment (at 56℃ for 30 min) and after acid treatment (pH 2 for 24 h).[15] The MAF activity of rainbow trout IFN-γ-like factor showed similar sensitivities to heating (60℃) and acid (pH 2) as mammalian IFN-γ.[11] Mammalian IFN-γ are species specific. For example, human IFN-γ can only affect human macrophages.[16] The present work suggests that the Con A-stimulated cell supernatants from Japanese parrotfish contains a IFN-γ-like factor.

Pmϕ monolayers are commonly contaminated by some lymphocytes. If stimulated cell supernatant contains free Con A, during the phagocytosis and

peroxidase production assay, Pmϕ could be activated by MAF from contaminated lymphocytes. However, we used Con A bound to Sepharose 4B beads for stimulating the lymphocytes, so that our Con A-stimulated supernatant did not contain free Con A. Accordingly, this lymphocyte-macrophage assay might be of benefit in a host defence test of cultured fish against viral and/or bacterial infect.

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イシダイのマイトジェン刺激リンパ球による マクロファージ活性因子の産生と本因子の特性

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イシダイの脾臓細胞中のリンパ球をコンカナバリン A（Con A）セファロース4B とともに48時間培養し，その上清によるマクロファージ活性化を検討し，以下の結果を得た。Con A 培養上清で培養したイシダイ腹腔マクロファージの貪食能と細胞内のペルオキシダーゼ活性の両機能は共に亢進していた。イシダイ Con A 培養上清をホ乳類で INF-γ 失活処理とされている熱（56℃）あるいは酸（pH2）処理を行ったところ，マクロファージの活性化が消失した。イシダイ Con A 培養上清の腹腔マクロファージ活性化の種特異性について，マウス腹腔マクロファージを用いて検討したところ，後者は活性化能を持たず，イシダイ Con A 培養上清の種特異性が存在した。

以上のことより，養殖イシダイ脾臓細胞中のリンパ球は，Con A で培養すると INF-γ を産生すると推察され，イシダイの魚体内で INF-γ，マクロファージの免疫防御機構の存在が示唆された。

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