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Analysis of IL-2-like factor in lymphocyte culture supernatant of olive flounder, *Paralichthys oliveaceus**

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Abstract To study immune mechanism of fish lymphocyte we performed a proliferation assay and ELISA using monoclonal antibody against human IL-2. The result showed that an interleukin-2 (IL-2)-like factor was detected in the supernatant of plant haemoglutinin (PHA)-stimulated lymphocyte culture from peripheral blood, spleen and head kidney of olive flounder, *Paralichthys olivaceus*. The quantities of IL-2-like factor in the supernatant from different lymphoid tissues were quite different. The IL-2 like factor in the supernatant from cultured head kidney lymphocytes was much higher than those of peripheral blood lymphocytes and spleen lymphocytes ($P<0.01$). The IL-2 activity was found in either mouse thymocyte proliferation assay or flounder head kidney lymphocyte proliferation assay and shown to have obvious enhancing effect on proliferation of the above two types of cell. The recombinant human IL-2 (rhIL-2) was able to stimulate flounder thymocyte proliferation and used to detect the IL-2 receptor (IL-2R) on the surface of flounder lymphocyte. The cross-reaction between the lymphocytes of flounder peripheral blood and CD25(IL-2R) was detected with flow cytometry and shown that the percentage of CD25-positive cell in peripheral blood was 7.74±0.67%.

Key words: Interleukin-2, IL-2 receptor, olive flounder, lymphocyte

1 INTRODUCTION

Interleukin-2 (IL-2) is a major immunoregulatory molecule produced by helper T cell and other related cells; it stimulates the activities of T-cell, NK cell and other cells and plays an important role in the process of transcription, translation, signal transduction and immunological regulation of nervous system. A total of 20000 lymphokines had been recognized at gene level in fish. But to the best knowledge of the authors, there has been a lack of reports of aquatic cytokine's structure and function.

In fish, the humoral immune response *in vivo* has been shown to possess functional equivalents to the interactive T and B cell systems of mammals. The expression of them is regulated by modulation in ambient temperature and physicochemical properties of the antigen, allowing complete separation between the functions of antibody synthesis, help and suppression in the intact animal. Caspi and Avtalion (1984) used mitogen to stimulate carp's leukocyte to induce IL-2 activity. Graham and Secombes (1990) also reported his finding of IL-2-like activities in mitogen-stimulated leukocyte culture supernatant. Xia and Kusuda (1993) used PHA and ConA to stimulate leukocytes in MLR assay and gave some evidence for the existence of an IL-2-like factor in eel. Luft et al. (1991) found a growth-promoting factor for peripheral blood lymphocytes in leikocyte culture supernatant in channel catfish. Guo (2001) found an IL-2-like factor in the supernatant of ConA-stimulated splenocyte culture of grass carp. Tamai et al. (1993) cloned the IL-2 gene of the flounder and proved that IL-2 genes of olive flounder share 42% homology to that of human. To study immune mechanism of fish lymphocyte, the activities of IL-2-like factor in the culture supernatant of flounder lymphocyte were examined and analyzed in this paper.

2 MATERIAL AND METHODS

2.1 Materials

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The consistency of human lymphocytes separating medium (hLSM) that purchased from Shanghai Jingsheng Biotech Co., Ltd, was adjusted to 1.083g/ml according to the consistency of peripheral blood cell. Recombinant IL-2 of human leukocyte (rIL-2) was purchased from Xinhua Hightech Co., Ltd. Mouse anti-human CD25 (IL-2 R) monoclonal antibody and FITC labeled anti-mouse IgG were purchased from France Immunotech. Human IL-2 ELISA kit was purchased from Jingmei Biotech Co., Ltd.

2.2 Preparation of IL-2 containing supernatant of olive flounder

Olive flounder peripheral blood was taken from caudal vein using a syringe half full of medium to prevent blood aggregation. The blood cells were laid on adjusted hLSM and centrifuged at 1000 rpm for 40 min at room temperature. The resulting band of leukocytes was drawn and washed several times with Hank's medium. Leukocytes (2×10^6 cells/ml) were cultured in M1640 containing 20% NBS at 22 ℃ for 4 hours, then cultured in M1640 containing 20% NBS and PHA(10μg/ml) at 22 ℃ for 72 hours. The supernatant was collected by centrifugation, filtered and stored at −30℃ for proliferation assay.

2.3 Mouse thymocytes proliferation assay

Samples of thymocytes from 4-8-weeks- old Kunming mice were assayed for IL-2 activities, as described by Guo (2001). Thymocyte proliferation induced by olive flounder IL-2-containing lymphocyte culture supernatant (at 12,1:4,1:8,1:16,1:32 and 1:64 dilution) and by rhIL-2 (1:64) was detected (Beck and Habicht, 1986).

2.4 Head kidney lymphocyte and thymocyte proliferation assay

Stimulations of head kidney lymphocytes and thymocytes were used for analyzing IL-2-like factor. Head kidney lymphocyte proliferation and thymocyte proliferation induced by olive flounder IL-2-containing supernatant (at 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilution), rhIL-2 (1:64) and PHA (10μg/ml) were measured in the procedure described by Guo (2001).

2.5 Quantity of IL-2-like factor in the supernatant

The quantity of IL-2-like factor in the lymphocyte supernatant of the flounder was detected using human IL-2 ELISA kit.

2.6 Cross reaction between the peripheral blood lymphocytes of flounder and the monoclonal antibody of leukocyte antigen CD25

Olive flounder peripheral blood was taken from caudal vein using a syringe half full of medium to prevent blood aggregation. Mouse anti-human CD25 monoclonal antibody was used to examine the surface antigen of lymphocyte. 100 μl of diluted blood sample was added to 20μl of FITC-labeled monoclonal antibody which against surface antigen to be examined, mixed rotatively for 15 min, then hold still in darkness for 1 min. Coulter (Coulter, USA) Q-prep pretreatment machine was used to breakdown red blood cell. After 35 cycles of treatment, the surface antigen was analyzed by Coulter flow cytometer (Coulter, USA).

3 RESULTS

3.1 Thymocyte proliferation of mouse

The IL-2-containing lymphocyte culture supernatants of flounder have evident effect on mouse thymocytes proliferation ($P<0.01$). As shown in the Fig.1, IL-2 has no significantly different effect on

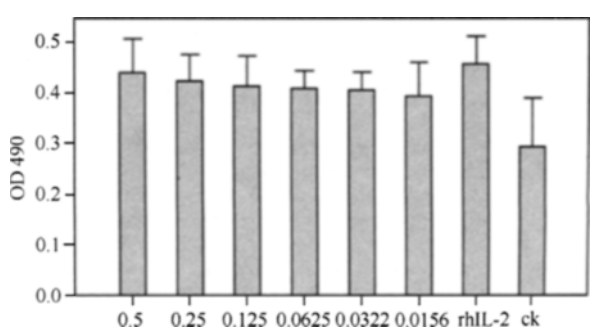

Fig.1 Mouse thymocyte proliferation induced by IL-2 containing supernatant of olive flounder, *Paralichthys olivaceus* (0.5, 0.25, 0.125, 0.06 25, 0.033 2, 0.015 6, for the IL-2 containing supernatant samples at 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 dilution), rhIL-2 (recombinant IL-2 of human) and the control (peripheral blood lymphocytes culture supernatant without PHA treatment)

thymocyte proliferation of mouse when compared with recombinant IL-2 of human leukocyte (rIL-2).

3.2 Flounder thymocyte proliferation and head kidney lymphocyte proliferation

The IL-2 containing flounder lymphocytes culture supernatant has evident effect on both flounder thymocyte and head kidney lymphocyte proliferation ($P<0.01$), as shown in Fig.2 and Fig.3.

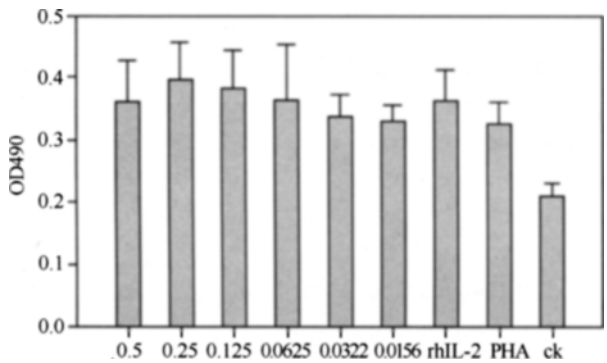

Fig.2 Head kidney lymphocyte proliferation induced by IL-2-containing supernatant of cultured lymphocytes of olive flounder, *Paralichthys olivaceus* (0.5, 0.25, 0.125, 0.0625, 0.0332, 0.0156, for IL-2 containing supernantant samples at 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilution), rhIL-2 (recombinant IL-2 of human), PHA and the control (peripheral bloody lymphocytes culture supernatant without PHA treatment)

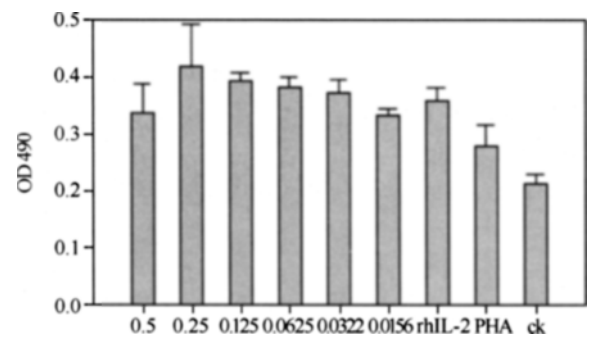

Fig.3 Thymocytes proliferation induced by IL-2-containing supernatant of cultured lymphocytes of olive flounder, *paralichthys olivaceus* (0.5, 0.25, 0.125, 0.0625, 0.0332, 0.0156, for IL-2 containing supernantant samples at 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilution), rhIL-2 (recombinant IL-2 of human), PHA and the control (peripheral bloody lymphocytes culture supernatant without PHA treatment)

3.3 The quantity of IL-2-like factor in the supernatant

The IL-2-like factor in the lymphocyte culture supernatent of flounder was detected by using human IL-2 ELISA kit. The result showed that there is no significant difference in the quantities of mitogen-stimulated lymphocytes from peripheral blood and spleen ($P>0.05$). The quantities of IL-2-like factor in lymphocyte supernatant of head kidney are much higher than those of peripheral blood and spleen (Fig.4).

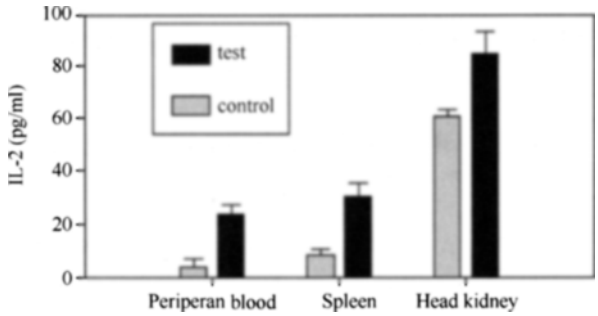

Fig.4 The quantities of IL-2-like factor in supernatant of cultured lymphocytes from peripheral blood, spleen and head kidney of olive flounder, *Paralichthys olivaceus*. (The control supernatant samples of lymphocytes were cultured in the same condition without PHA)

4 DISCUSSION

IL-2 is formally known as T-cell growth factor in recognition of its ability to induce the proliferation of T-cell (Rast et al., 1995). In mammals, IL-2 is produced primarily by helper $CD4^+$ T-cells although other subsets of T cells may also be involved in IL-2 stimaulation of other cells in the immune system such as NK cells. At present, no reagent is available to identify $CD4^+$/ $CD8^+$/$CD3^+$ molecules in fish. It is unsure what respective effects of $CD4^+$ and $CD8^+$ in fish would be (Hashitmoto et al., 1990, 1992; Fisher et al., 2003; Okamura et al., 1993). It is shown in this study that the olive flounder, *Paralichthys olivaceus* lymphocytes secrete IL-2 like factor. The IL-2 activity was found in mitogen (PHA)-stimulated lymphocyte from peripheral blood, spleen and head kidney cultured *in vitro*. Function of IL-2 in the olive flounder is unknown, although it may have activities similar to those of vertebrate Il-2. In fish, soluble factors with IL-2-like activity were detected following T-cell activation *in vitro* (Caspi and Avtalion, 1984). IL-2 stimulates other cells in the immune system such as NK cells (Shen et al., 2002). It was found that in a long term culture lines of channel catfish T cells were immor-

talized by stimulation with phorbol ester and ionophore (Lin et al., 1992). In this study, IL-2-like activity was found in both mouse thymocyte proliferation assay and flounder head kidney lymphocyte proliferation assay, and showed obvious promotive effect on the proliferation of the above cells. It is suggested that this IL-2 like factor of flounder have many properties in common with those of mammalian IL-2 factor.

The quantities of IL-2-like factor in the supernatant from different lymphoid tissues were quite different. The quantities of IL-2 like factor in the supernatant from cultured head kidney lymphocytes were significantly higher than those in peripheral blood lymphocytes and spleen lymphocytes. It is suggested that there are different T-cell subsets in different tissues, and that IL-2 secreting cells are mainly in head kidney. A cultured supernatant of PHA-prestimulated PBL containing IL-2-like activity decreased spontaneous apoptosis in both T and B cells, but did not affect cortisol-induced apoptosis in B cells. Apoptosis in thrombocytes was unaffected by either mitogens, cortisol, or lymphocyte supernatant. The difference between mammalian and fish leukocyte sensitivity to cortisol is discussed in terms of differences in immune response of mammals and fish (Verburg-Van-Kemenade et al., 1999). Although existence of helper cell activity and the corresponding function of cytotoxic T cell/Ts are well known, recognition of the cells that are responsible for their functions in the fish specific immune responses is still difficult, as classification of leukocytes primarily relies on morphological parameters. In mammalian immunology, monoclonal antibodies (MAbs) against cell surface molecules are important tools to identify different leukocytes and a similar availability of MAbs to fish leukocytes will facilitate the study of the fish immune system considerably.

Result revealed an IL-2R-like structure on the surface of flounder lymphocyte and the percentage of CD25-positive cell in peripheral blood is $7.74 \pm 0.67\%$. It indicated that there are human IL-2R-like structures on the surface of flounder lymphocyte. This IL-2 like factor of flounder seems very similar to mammalian IL-2 in molecular structure and function (Tamai et al., 1992; 1993). The authors believed that the interaction of IL-2 and IL-2R is highly conserved in the evolution of animal immunity. Further study of the IL-2-like factor would be essentially needed for exploring the evolution.

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