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Inhibition of Cyclosporine A or rapamycin on T lymphocyte counts and the influence on the immune responses of B lymphocytes in flounder (*Paralichthys olivaceus*)

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ABSTRACT

In acquired immunity, T lymphocytes regulate the immune responses of B lymphocytes, including the IgM^+ B lymphocyte counts and antibody production. In this paper, Cyclosporine A (CsA) and Rapamycin (RaPa) were used, and their inhibition on T lymphocytes and immune responses of B lymphocytes in flounder (*Paralichthys olivaceus*) were investigated. Flounder was injected with Keyhole Limpet Hemocyanin (KLH), a mixture of KLH and CsA (KLH + CsA), or a mixture of KLH and RaPa (KLH + RaPa). Then, the proportions of T and IgM^+ B lymphocytes (PT and PB) in peripheral blood leukocytes (PBL) were analysed by flow cytometry (FCM), total antibodies (TA) and KLH specific antibodies (KA) in serum were measured by ELISA, and expression of 9 immune-related genes in the spleen and kidneys were determined using q-PCR. On the other hand, the PBL culture was treated with Concanavalin A (ConA), a mixture of ConA and CsA, and a mixture of ConA and RaPa. Then the PT and PB were measured, and the cell proliferation was examined using the MTT method. The results showed that the PT peaked on the 5^{th} day in the KLH group, KLH + CsA group and KLH + RaPa group. The maximum inhibition rates (MIR) of CsA and RaPa were 27.44% ± 0.50% and 21.37% ± 2.06%, respectively. The PB peaked at the 5^{th} week, and the MIR of CsA and RaPa were 44.51% ± 1.36% and 33.3% ± 0.65%, respectively. The KA and TA peaked at the 5^{th} week. The MIR of CsA and RaPa on TA were 40.31% ± 1.59% and 32.96% ± 2.21%, respectively, and were 27.77% ± 2.02% and 23.41% ± 1.08% for KA, respectively. Nine immune-related genes had significantly lower expression in the KLH + CsA group and KLH + RaPa group compared to the KLH group. The proliferation of the PBL culture was inhibited by CsA or RaPa, and the inhibition rate of CsA and RaPa for PT was 18.14% ± 1.08% and 17.88% ± 1.02%, respectively, and the inhibition rates for PB were 3.03% ± 0.57% and 2.95% ± 0.53%, respectively. The results demonstrated that T lymphocytes counts were inhibited by CsA and RaPa, followed by suppression of IgM^+ B lymphocytes and antibody production, which suggests that T lymphocytes regulate the immune response of B lymphocytes in flounder.

1. Introduction

In fish adaptive immunity, T lymphocytes participate in the cellular response [1–3]. However, B lymphocytes are involved in humoral immunity by secreting antibodies [4]. In recent years, genes related to T lymphocytes and B lymphocytes, immunoglobulin (Ig), and antibody production in rainbow trout [5], flounder [6], and channel catfish [7] have been reported. Detection techniques at the cellular and genetic levels in T, B lymphocytes were developed, but the connection between T and B lymphocytes and the immune response was not clear. Cyclosporine A (CsA) and Rapamycin (RaPa) are common immunosuppressants found on lymphocytes in mammals [8,9]. Similarly, T cell proliferation was inhibited by CsA under *in vitro* conditions in channel catfish, but no inhibition was found in B lymphocytes [10]. Our previous work included the production of monoclonal antibodies against flounder IgM [11], and then the recombinant protein and the antibodies for the flounder (*Paralichthys olivaceus*) T lymphocyte surface

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molecule, CD3 [12,13]. There were specific probes for T lymphocyte counts and IgM^+ B lymphocytes as well as antibody detection. Therefore, in this paper, CsA and RaPa were used, and their inhibition in T lymphocytes counts and the immune responses of B lymphocytes in flounder (*Paralichthys olivaceus*) were investigated. The purpose of this study was to determine whether T lymphocytes regulate the immune response of B lymphocytes in flounder.

2. Materials and methods

2.1. Fish

Apparently healthy flounder (*Paralichthys olivaceus*) (weight: 15–25 g, body length: 10–15 cm) were obtained from a farm in RiZhao, Shandong province, China. A total of 800 fish were kept in the laboratory, supplied with running seawater at 20–22 °C, and fed daily with dry food pellets. After they were acclimated to laboratory conditions for 2 weeks, they were used for the following experiments.

2.2. Reagents and antibodies

Keyhole Limpet Hemocyanin (KLH, cat No. H7017), Cyclosporine A (CsA, cat No. C3662), Concanavalin A (Con A, cat No. C2272) and Rapamycin (RaPa, cat No. V900930) were purchased from Sigma, USA. KLH was dissolved at 4 mg/mL with PBS, CsA or RaPa, which was first dissolved at 50 mg/mL with DMSO, and ConA was dissolved into 5 mg/mL with PBS, and then the solutions were used in the following experiments.

Mouse anti-flounder IgM monoclonal antibodies (FIgM-Mab, 1:1000 diluted in PBS) were produced previously in our laboratory and their specificity to IgM^+ B cells was verified with Flow cytometry (FCM) and an immunofluorescence assay [11], and in this study, the ascite fluids were used in FCM, an Enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence assay (IFA). The rabbit anti-flounder CD3 polyclonal antibodies (FCD3-Pab) were produced in our lab [12,13], and the specificity for CD3 was verified using co-immunoprecipitation, Western blotting and mass spectroscopy. In this study, a dilution of 1:500 in PBS was used in IFA and FCM.

2.3. CsA and RaPa injection experiments and sampling

CsA and RaPa were diluted to 2 mg/mL and 0.4 mg/mL with PBS, respectively. Fish were randomly divided into four groups with 150 fish in each group. They were injected in their abdominal cavities with 100 μL 2 mg/mL KLH, 50 μL 4 mg/mL KLH and 50 μL 2 mg/m CsA, 50 μL 4 mg/m KLH and 50 μL 0.4 mg/mL RaPa. The control group was injected with 100 μL PBS.

Six individuals in each group were anesthetized, and the peripheral blood was collected at 1d, 3d, 5d, 7d, 14d, 21d, 28d, 35d and 42d after injection. The isolation protocol of PBL was performed according to a previously reported method [14,15]. Briefly, blood was drawn from the caudal vein, diluted 1:1 in solution (65% RPMI-1640 containing 20 IU mL-1 heparin, 0.1% w/v NaN3 and 1% w/v BSA), and stored at 4 °C for 1 h, then centrifuged at 100 g for 10 min. Then they were applied on the Percoll density media between 1.020 and 1.070 g/cm^3 and centrifuged at 840 g for 30 min. The cell layers at the Percoll interface were collected as leukocytes and washed 3 times with PBS containing 5% (v/v) new-born calf serum by centrifugation at 640*g*, and then were suspended in PBS. Then, the isolated PBL were adjusted to 1.0×10^6 cells mL^{-1}, and the percentage of T lymphocytes and IgM^+ B lymphocytes (PT and PB) in PBL were tested using FCM. Three individuals in each group were anesthetized, and the serum was collected at 1w, 2w, 3w, 4w, 5w and 6w post-injection, respectively. The isolation protocol of serum was performed according to a previously reported method [16]. Then, the levels of total antibodies (TA) and KLH-specific antibodies (KA) in the serum were tested using ELISA. The spleen and the kidney were randomly sampled from 3 fishes at 12 h, 24 h, 36 h, 48 h, 3d, 5d, 7d and 14d post-injection. Additionally, total RNA extraction followed the Trizol method, and the RNA integrity was detected using 1% agarose gel electrophoresis. RNA concentration and purity were detected with a trace nucleic acid analyser (NanoDrop 8000, Thermo, USA). Then, cDNA was synthesized using the PrimeScript™ reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. The cDNA samples were stored at −20 °C until they were used, and then the immune-related gene expression was quantified using q-PCR.

2.4. Addition of CsA and RaPa to cultured peripheral blood leukocytes

CsA, RaPa and ConA were diluted to 40 μg/mL, 8 μg/mL and 60 μg/mL with L-15 medium (Gibco, USA), respectively. Healthy flounder PBL were suspended with L-15 in a 5.0×10^6 cells mL^{-1}, 100 μl PBL suspension that was applied to each well of a 96-well plate and cultured at 22 °C for 12 h. Then 100 μL of 30 μg/mL ConA, 50 μL of 60 μg/mL ConA and 50 μL of 40 μg/mL CsA, 50 μL of 60 μg/mL ConA and 50 μL of 8 μg/mL RaPa were added to each well, respectively. After the cells were cultured for 48 h, the PBL were collected, the PT and PB in cultured PBL were tested using FCM and cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT) method.

2.5. Flow cytometry, immunofluorescence assay, and Giemsa staining

The PBL was incubated with both FIgM-Mab and FCD3-Pab for 1 h at 37 °C. Subsequently, the cells were washed 3 times with PBS containing 5% (V/V) New-born Calf Serum by centrifugation at 640*g* for 5 min, then the cells were incubated with both goat-*anti*-mouse Ig-FITC (1:256, Sigma, USA) and goat-*anti*-rabbit Ig-Alexa Fluor® 647 (1:1000, Thermo Fisher Scientific, USA) for 1 h in the dark at 37 °C and washed again. Next, the cells were resuspended in 1 mL of PBS and analysed by Accuri C6 flow cytometer (BD, USA). Side- and forward-scatter (SSC and FSC) parameters were used to determine cell granularity and cell size, respectively. Fluorescent

Table 1
The specific primers used in quantitative real-time PCR.

Primer No.	Primer name	Primer sequence	Source
1	18SRNA-F	5′-GGTCTGTGATGCCCTTAGATGTC- 3′	EF126037
2	18SRNA-R	5′-AGTGGGGTTCAGCGGGTTAC- 3′	
3	INF-γ-F	5′-TGTCAGGTCAGAGGATCACACAT- 3′	AB435093
4	INF-γ-R	5′-GCAGGAGGTTCTGGATGGTTT- 3′	
5	CD40-F	5′-TGGTGGTGATGCTGTGCA- 3′	AB081752
6	CD40-R	5′-CCACACTCTGCGCACTGA- 3′	
7	MHCI-F	5′-AGACCACAGGCTGTTATCACCA- 3′	AB126921
8	MHCI-R	5′-TCTTCCCATGCTCCACGAA- 3′	
9	MHCII-F	5′-ACAGGGACGGAACTTATCAACG- 3′	AY997530
10	MHCII-R	5′-TCATCGGACTGGAGGGAGG- 3′	
11	CD4-1-F	5′-CCAGTGGTCCCCACCTAAAA- 3′	AB643634
12	CD4-1-R	5′-CACTTCTGGGACGGTGAGATG- 3′	
13	CD4-2-F	5′-CACAGCGAGGACGTCAGAAA- 3′	AB640684
14	CD4-2-R	5′-TCTCTCCCATCACTCCTTTAGCA- 3′	
15	CD8-α-F	5′-CCTCTCCCCATACATTGATTCC- 3′	AB082957
16	CD8-α-R	5′-CCGAGCTTTGCTGAAGGACTT- 3′	
17	CD8-β-F	5′-GATGACACTCAAACCTCCAGTCAA- 3′	AB643633
18	CD8-β-R	5′-GCCATCCTGTGCAAAATTCTTC- 3′	
19	IgM-F	5′-AAGTCCACAAATTACCCTCCAA- 3′	AB052744
20	IgM-R	5′-TTCTCGCTTTTATGTTCCTCAG- 3′	