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Production of Monoclonal Antibodies against Yellowtail Leucocyte

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Abstract: BALB/c mouse was immunized with yellowtail peripheral blood leucocytes (PBLs) and spleen cells removed from the immunized mouse were fused with myeloma by using polyethyleneglycol method. Antibody producing hybridomas were screened by ELISA using mixed leucocytes as target antigen. A total of 8 hybridomas (culture no. AB-2, AB-10, BB-11, BC-5, BC-6, CF-11, DG-10 and EG-11) were obtained by single cell cloning. Monoclonal antibodies (MAbs) produced in the culture supernatant of cloned hybridomas were used for further characterization. Imunofluorescent stain using FITC labeled rabbit secondary antibody revealed that MAbs AB-2, AB-10 and EG-11 recognized lymphocyte and MAbs BC-5, CF-11 and DG-10 recognized granulocyte or monocyte. MAbs BC-6 and BB-11 reacted to yellowtail lymphocyte which bore immunoglobulin of yellowtail.

Key words: Monoclonal antibody, yellowtail, leucocyte, classification of leucocytes.

INTRODUCTION

Monoclonal antibody (MAb) has been used in variable scientific fields for its precise function to identify antigens. However in the field of fish immunology, the use of MAb to analyze fish immune system is not yet established (Loob and clean, 1982; DeLuca *et al.*, 1983, Secombes *et al.*, 1983 and Navarro *et al.*, 1993). Fish leucocytes have been classified on the basis of cell morphology (Ellis, 1977). Recently, attempts were made to charaterize leucocyte subpopulations by using MAbs against specific membrane antigens of carp (Secombes *et al.*, 1983), rainbow trout (DeLuca *et al.*, 1983), channel catfish (Lobb and Clem, 1982) and sea bream (Navarro *et al.*, 1993) enabling identification to be B lymphocyte. Production of MAbs against leucocytes other than B lymphocyte have also been reported for different fishes (Evans *et al.*, 1988; Secombes *et al.*, 1983; Anisworth *et al.*, 1990; Bly *et al.*, 1990; Scapigliati *et al.*, 1995 and Nakayasu *et al.*, 1995). Separation of leucocyte subpopulations has been performed mainly by density gradient centrifugation. MAbs raised against specific subpopulations enable also to establish such means to separate leucocyte subpopulations.

Yellowtail *Seriola quinqueradiata* is one of the most important marine fish for aquaculture in Japan. Development of vaccines against infectious disease and characterization on immune response of this fish have been expected. However, by the reason that cellular profiles of immune system of this fish is not yet investigated enough, it is difficult to analyze reaction after immunization. To solve the problems, it is important to recognize functions of each subpopulation of leucocytes or lymphocytes. The present study to produce MAbs against yellowtail leucocyte subpopulations was conducted to establish identification means for the analysis of those immunopotent cells.

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MATERIALS AND METHODS

Fish

Yellowtail weighing about 150 g were obtained from a fish farm in Kochi Prefecture and transported to the laboratory of Usa Marine Biological Institute, where they were maintained in 600 *l* tanks with flowthrough sea water. Fish were fed commercial food pellet until use.

Antigen

Blood was collected with a heparinized syringe from the heart of yellowtail, diluted in phosphate buffered saline (PBS) and overlaid onto 3 m*l* Ficoll-Paque (Pharmacia). After centrifugation at 400 × *g* for 30 min, peripheral blood leucocytes (PBLs) were collected from the interface layer between Ficoll-Paque and diluted blood and washed twice in PBS by centrifugation at 300 × *g* for 10 min. Siliconized pastur pipettes and glass tubes were used while PBL separation.

Production of MAb

Three BALB/c mice of 4 weeks old were injected intraperitonealy with 1×10^7 cells of yellowtail PBLs for 4 times at 1 week intervals without adjuvant. Three days after the last injection, the spleen was taken from the immunized mice and spleen cells were suspended in RPMI-1640 medium (Nissui) without fetal bovine serum (FBS). The spleen cell suspension was mixed with BALB/c mouse myeloma cell line P3 × 63-Ag.8.653 and they were fused with 50% w/v polyethyleneglycol (PEG) solution (Hybri max, Sigma) according to the method of Osamune and Terada (1990). Hybridomas were washed by centrifugation (300 × *g*, 5 min) and cultured in hypoxanthine-aminopterin-thymidine (HAT) medium containing 5% Briclone (Dai Nihon Seiyaku) in 96 well microplate at 37°C in a humidified 5% CO_2 atmosphere. HAT medium was exchanged 100 μl every 3 days. After 14 days from cell fusion, medium was replaced to hypoxanthine-thymidine (HT) medium gradually and hybridoma supernatant was tested for production of antibody against PBL by ELISA. The 96 well flat bottom microtiter plate was coated with 0.1% poly-L-lysine solution to adhere PBLs. 100 μl of concentrated PBL suspension was added to each well and incubated at 4°C for overnight to attach. Afterwards 100 μl of 1% bovine serum albumine in PBS was added for blocking, incubated at room temperature for 1 h and washed 3 times with PBS. After washing the wells were incubated at 37°C for 2 h with 50 μl of hybridoma culture supernatant. The plate was washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) and added 100 μl of a 1:1,000 diluted rabbit anti-mouse immunoglobulin G (IgG) conjugated with peroxidase then incubated at 37°C for 1 h. The plate was washed 3 times PBS-T and incubated at room temperature (about 25°C) for 30 min with 100 μl of 0.005% H_2O_2 and 0.25% *o*-phenylenediamine in citric acid-phosphate buffer (citric acid·H_2O 1.02 g, $Na_2HPO_4 \cdot 12H_2O$ 3.69 g, /100 m*l* water, pH 5). The reaction was stopped with 1 N H_2SO_4 and absorbance was determined at 492 nm by a micro plate reader (MPR A4*i*, Tosoh). Antibody-positive was evaluated by detecting 3 times greater absorbance than the control well. Hybridoma in HT medium was diluted and dispensed into 96 well micro plate so that each well contained single cell. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 until proliferation of the cell was observed.

Immunofluorescent stain

PBL suspension separated by Ficoll-Paque was smeared on a slide glass and dried. After dry the cells were fixed with 100, 90 then 70% ethanol each for 5 min and washed by immersing the slide glass in distilled water for 3 min. The smear was blocked with 1% skim milk in PBS

for 30 min. After incubation the slide glass was washed 3 times in PBS. After wash hybridoma supernatant was put on the smear and incubated at room temperature for 1 h. After incubation it was washed 3 times in PBS. Fluorescent isothyocyanite conjugated rabbit anti-mouse IgG was put on the smear and incubated at room temperature in a dark for 1 h. After incubation the slide was washed 3 times in PBS and mounted with 70% glycerol and coverslip then observed by a fluorescence microcope.

RESULTS

Production of MAb

From day 14 after fusion, cultured hybridoma supernatant turned yellow showing growth of the cells in more than 95% wells. Unfused spleen cells were observed until 14th day, however after 18 days all of them died. Antibody producing hybridoma was screened by ELISA 3 times on 14, 18 and 22th day after cell fusion by monitoring positive to yellowtail PBL antigen and negative to erythrocyte antigen. At last 5 positive hybridoma cultures were obtained. 21 days after the cloning started 8 cultures were obtained as cloned hybridoma (Table 1).

Table 1. Cloned hybridoma cultures which produced specific antibody against yellowtail leucocyte

Culture No.	ELISA titer*1 of culture supernatant against	
	Leucocyte	Erythrocyte
AB-2	0.450	0.265
AB-10	0.497	0.277
BB-11	0.499	0.266
BC-5	0.461	0.208
BC-6	0.905	0.274
CF-11	0.454	0.267
DG-10	0.519	0.295
DG-11	0.678	0.288
Control*2	0.137	0.220

*1 Absorbance at 492 nm.

*2 HAT medium was used instead of hybridoma supernatant.

Immunoflourescent stain

The supernatants of 8 cloned hybridomas were used for immunofluorescent stain to detect MAb against yellowtail PBL. The results showed specific reaction to total PBL. However, each supernatant recognized different kind of cells as shown in Fig. 1. Out of 8 supernatants of cloned hybridomas two sets of supernatants, a set of AB-2 and AB-10 and another set of BC-6 and BB-11 recognized similar cells, respectively. The former stained small and round cells. The later set of supernatant stained cell surface antigen of relatively small lymphocyte-like cells. The supernatant of BC-5 stained large irregular formed cells. The supernatant of CF-11 stained round or irregular formed cells. The supernatant of DG-10 stained the cytoplasm of large round cells which had vacuoles in side. The supernatant of EG-11 stained round lymphocyte-like cells.

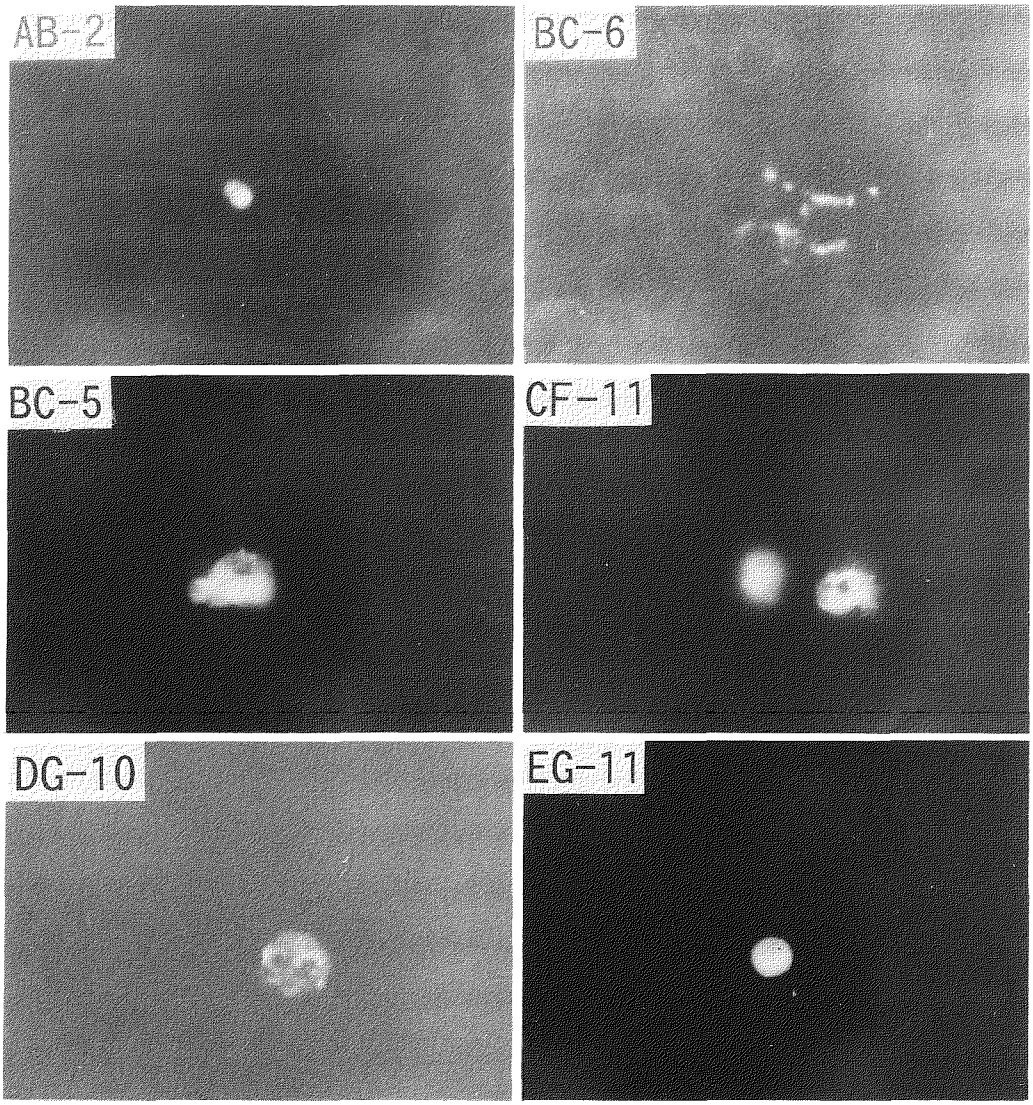

Fig. 1. Immunofluorescence of yellowtail leucocytes stained with supernatants from 8 hybridoma cultures.

DISCUSSION

By screening 3 times on 14, 18 and 22 days after cell fusion, only 5 hybridoma cultures were obtained showing positive to yellowtail PBL antigen and negative to erythrocyte antigen. The low percentage of yield may be attributed to false detection of the antibody produced by unfused spleen cells which was accumulated in the culture supernatant or to the fragility of the antibody producing hybridoma. As it had been expected, single cell cloning resulted very low positive rate of growth. Nevertheless, 8 cultures of cloned hybridoma were established.

The immunofluorescent staining showed that supernatants of cloned 8 hybridomas reacted to yellowtail PBLs. The supernatants of AB-2 and AB-10 reacted to similar cells which show relatively small and round shape same to small lymphocyte. The supernatants of BC-5, CF-11

and DG-10 reacted to relatively large cells of irregular form. Those cells are presumed to be granulocyte or monocyte. The supernatant of EG-11 reacted to relatively large round cells similar to large lymphocyte. The supernatants of BB-11 and BC-5 reacted cells which type was not identified from the size and form. However, as the fluorescens was observed only at the surface of the cells. These cells were same to the surface immunoglobulin (SIg) bearing cell which was reported in yellowtail by Hamaguchi and Kusuda (1988). According to them, the target antigen which those MAbs recognize is presumed immunoglobulin of yellowtail and the SIg bearing cells are B lymphocytes.

From the results of the present study it is concluded that the 8 cloned hybridomas were established which produce specific MAbs recognizing different type cells of yellowtail leucocytes. However, the cell types which can be reacted with MAbs were identified roughly. In order to utilize the MAbs for further study, it is necessary to identify the cell types and target antigen more precisely.

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