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Production, characterization and application of monoclonal antibody against immunoglobulin D heavy chain of flounder (*Paralichthys olivaceus*)

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ABSTRACT

Immunoglobulin D (IgD) is considered to be an enigmatic Ig molecule because of the lack understanding of its immunological functions. In the present study, a partial δ region of the flounder IgD was recombinantly expressed, purified and used as an immunogen to produce monoclonal antibodies (MAbs) against the H chain of flounder IgD. After fusion, a total of 97 hybridomas were generated and observed under an inverted microscope One of the hybridomas, designated 5G7, gave strong positive results in an indirect enzyme-linked immunosorbent assay (ELISA) and was cloned and subcloned by limiting dilution. Western blot analysis showed that MAb 5G7 could specifically recognize a 118 kDa protein from peripheral blood lymphocytes (PBLs), which was identified to be the H chain of flounder IgD by mass spectrometric analysis. Indirect immunofluorescence assay tests (IIFAT) showed that specific fluorescence signals were observed on the membranes of the PBLs, which suggests that MAb 5G7 could recognize the membrane-bound IgD molecule. Moreover, only the subset of IgD+/IgM + B cells were observed in the PBLs of healthy flounder when tested by flow cytometry analysis. Consistent with the results of flow cytometry, a double immunofluorescence assay test (DIFAT) showed that the positive lymphocytes were stained with both green and red fluorescence signals, which represent the IgM+/IgD + lymphocytes subset. These results demonstrate that the produced MAb 5G7 could specifically recognize the flounder IgD, which provides a useful tool to study the functions of flounder IgD.

1. Introduction

Immunoglobulins (Igs) are important molecules in the innate and adaptive immune systems of jawed vertebrates. Igs are useful in protecting against a wide range of pathogens [1,2]. In mammals, five Ig isotypes were identified based on the constant domains of their H chains. In teleost fish, there are three major Ig isotypes: IgM, IgD and IgT, defined by the heavy chains μ, δ and τ, respectively. Among the three Ig types, IgM is the earliest identified Ig isotype, which presents as the main antibody in serum and is secreted into the mucus via the polymeric Ig receptor (pIgR) [3,4]. IgZ/T was found to play an important role in mucosal immunity similar to IgA in mammals [5,6]. In contrast, IgD has remained an enigmatic isotype since its discovery over 50 years ago. IgD is a primitive class of immunoglobulin present in most jawed vertebrates and conserved through evolution [7]. Initially, IgD was only detected as a membrane-bound form in teleosts, but a secreted form was subsequently discovered in catfish [8] and rainbow trout [9]. In recent decades, research on IgD in teleost fish has attracted much attention, and many IgD genes have been cloned and characterized, such as cod (*Gadus morhua*) [10,11], Japanese flounder (*Paralicthys olivaceus*) [12], fugu (*Fugu rubripes*) [13], Atlantic salmon (*Salmo salar*) [14] and zebrafish (*Danio rerio*) [15]. Teleosts demonstrate remarkable plasticity in their δ genomic arrangement, with many species possessing repeated blocks of exons encoding Cδ2–4 as well as duplicated δ loci [16–18].

To date, information regarding the immunological properties and biological functions of IgD is very limited. In mammals, IgD was reported to function as an antigen receptor optimized for efficient

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recruitment of B cells in antigen-driven responses [19]. Moreover, IgD can largely increase in B cells, in which IgM function is suppressed, suggesting that IgD is largely able to substitute for IgM functions [20]. In addition, previous studies have demonstrated that the association of secretory IgD with basophils and mast cells results in the production of antimicrobial factors and the enhancement of respiratory immune resistance [8,21]. Taken together, these facts demonstrate a crucial role for IgD in coordinating the immune surveillance of the host at the intersection of the innate and adaptive immune systems. Teleost IgD is thought to play similar roles to mammalian IgD in the immune system [22]. However, in teleost fish, the available information about IgD is mainly its gene structure and expression profile after stimulation [23,24]. Flounder is an economically important fish popularly cultured in Asian countries, and accumulating studies on its immune system have been conducted in recent years. The cDNA encoding flounder IgD has been cloned and analysed previously [12], though no further research was performed at the protein level.

In the present study, the conserved δ1−δ4 region was recombinantly expressed and purified, which was then used as an immunogen to produce MAbs against the H chain of flounder IgD. Furthermore, the produced MAbs were used to investigate the distribution of IgD and IgM molecules by the double immunofluorescence assay test (DIFAT), and the percentage of IgD+ and IgM + lymphocyte subsets were determined by flow cytometric immunofluorescence analysis (FCIA).

2. Materials and methods

2.1. Expression and purification of recombinant IgD

According to the gene sequence encoding the IgD heavy chain of flounder (GenBank No. AB052658), specific primers were designed to amplify the δ1−δ4 region of flounder IgD (F: 5′-CGGGATCCAAAAGTCGGGTTGTCTCTCC-3'; R: 5′-CCAAGCTTTGAGCAGAGGCTGATATTC-3′). The PCR product was purified and digested with specific restriction enzymes and subsequently ligated into the pET-28a vector to construct recombinant plasmids. The recombinant plasmid pET-28a-IgD was transformed into *E. coli* BL21 (DE3) cells. The transformant was cultured in LB medium to mid-logarithmic phase and induced by adding isopropyl β-D-1-thiogalactopyranoside. His-tagged rIgD was purified using His TrapTM HP Ni-Agarose (GE Healthcare China, Beijing, China) following the manufacturer's instruction. Subsequently, the purified protein was renatured in TBS glycerol buffer (50 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 6.0 M urea, reduced glutathione, oxidized glutathione, pH 8.0) by four dialysis steps, with each step being performed at 4 °C for at least 12 h. The protein was analysed by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. The concentration of protein was determined using the Bradford method.

2.2. Production of monoclonal antibodies

Three BALB/c mice were each immunized by intraperitoneal injection with 100 μL of 1 mg/mL rIgD emulsified with an equivalent volume of Freund's complete adjuvant (FCA, Sigma, St. Louis, MO, USA) on day 1. Two weeks later, a similar injection was administered using Freund's incomplete adjuvant (FIA, Sigma, St. Louis, MO, USA) instead of FCA. Then, booster injections were given twice by tail vein injection at 1-week intervals. Three days after the last injection, the mice were sacrificed. Spleen cells were collected from the immunized mice and fused with myeloma P3-X63-Ag8U1 cells using 50% polyethylene glycol 4000. The cells were distributed into 96-well culture plates (Costar) in GIT medium (Nihon Seiyaku Co., Japan) supplemented with 1% HAT (Gibco, USA), and the culture medium was changed every 3–5 days. After 12–14 days, the supernatants from the wells growing hybridomas were screened using an indirect enzyme-linked immunosorbent assay (ELISA). Hybridomas showing positive results were cloned by the method of limiting dilution three times, and the monoclonal antibodies were characterized by ELISA, western blotting, IFAT and FCIA.

2.3. Indirect ELISA

Wells of flat bottom microplates (96-wells, Costar) were coated with purified rIgD (10 μg/well) in 100 μL of phosphate buffered saline (PBS) and incubated overnight at 4 °C. The wells were washed three times with PBS containing 0.05% Tween-20 (PBST) and later blocked with 200 μL of 3% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. The plate was washed and 100 μL of hybridoma culture supernatant diluted 1:100 in PBS was added as a first antibody and incubated for 1 h at 37 °C. After washing, 100 μL of goat-anti-mouse Ig-alkaline phosphatase conjugate (Sigma, St. Louis, Mo, USA) diluted 1:4000 in PBS was added as a second antibody and incubated for 1 h at 37 °C. Following a final wash, 100 μL 0.1% (w/v) p-nitrophenyl phosphate (pNPP, Sigma, St. Louis, MO, USA) in 50 mM carbonate-bicarbonate buffer (pH 9.8) containing 0.5 mM $MgCl_2$ was added to each well and incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 μL of 2 M NaOH to each well and absorbance was measured with an automatic ELISA reader at 405 nm. As a negative control, the myeloma culture supernatant instead of the Mab culture supernatant was added as the first antibody. Each experiment was repeated in triplicate.

2.4. Western blotting and mass spectrometric analysis

Peripheral blood lymphocytes (PBLs) were isolated from healthy flounder by a discontinuous Percoll (GE Healthcare China, Beijing, China) gradient (1.020/1.070) in accordance with a method described previously [25]. After SDS-PAGE, rIgD and PBLs were transferred onto a PVDF membrane (Merck Millipore, Darmstadt, Germany). The membrane was blocked with PBS containing 3% BSA for 1 h, incubated with the MAb for 1 h at 37 °C and subsequently washed three times with PBST. Antibody binding was detected with goat-anti-mouse Ig-alkaline phosphatase conjugate diluted 1:4000 in PBS for 1 h at 37 °C followed by washing three times with PBST. Positive bands were stained with freshly prepared substrate solution (100 mM NaCl, 100 mM Tris and 5 mM $MgCl_2$, pH 9.5) containing nitroblue tetrazolium (NBT, Sigma, St. Louis, MO, USA) and 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma, St. Louis, MO, USA) for 5 min, after which the reaction was stopped by washing with distilled water. The immune-reactive proteins in PBLs were excised from polyacrylamide gels and analysed by an ABI5800 matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) assay system (Applied Biosystems, Beverly, MA, USA). Myeloma culture supernatant was used as the negative control.

2.5. Indirect immunofluorescence assay test (IIFAT)

For IIFAT, PBLs were isolated from healthy flounder as described above. The PBLs were resuspended with PBS containing 5% (V/V) newborn calf serum, then settled onto glass slides and fixed with acetone for 10 min and then stored at −20 °C after drying at room temperature. MAbs were added to glass slides and incubated for 1 h at 37 °C. After washing three times with PBST, the slides were incubated for 45 min at 37 °C with goat-anti-mouse Ig-FITC (1:256, Sigma, St. Louis, MO, USA). Finally, the slides were washed again with PBST and observed using a fluorescence microscope with