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Short Communication

Monoclonal antibody to serum immunoglobulins of *Clarias batrachus* and its application in immunoassays

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ABSTRACT

Serum immunoglobulins of *Clarias batrachus* (Cb-Ig) were purified by affinity chromatography using bovine serum albumin as capture ligand. Under reducing conditions in SDS-PAGE, Cb-Ig was composed of a heavy (H) chain (68.7 kDa) and two light (L) chains (27.4 and 26.3 kDa). Purified Cb-Ig was used to produce a monoclonal antibody (MAb) designated E4 MAb that belonged to IgG1 subclass. In Western blotting, this MAb showed binding to H chain of purified Cb-Ig and putative H chains in reduced sera of *C. batrachus*, *Clarias gariepinus* and *Heteropneustes fossilis*. However, no binding was observed with serum protein of *Labeo rohita* and *Channa striata*. Cross-reactivity of anti-Cb-Ig MAb was observed with serum of *C. batrachus*, *C. gariepinus* and *H. fossilis* in competitive ELISA. In immunoblotting of non-reduced Cb-Ig with E4 MAb, four bands assumed to be tetrameric, trimeric, dimeric and monomeric form were observed. In flow cytometric analysis of the gated lymphocytes, the number of surface Ig-positive (Ig+) cells in blood, spleen, kidney and thymus of *C. batrachus* was determined to be 50.1 ± 3.1, 55.1 ± 3.36, 42.4 ± 4.81 and 5.1 ± 0.89%, respectively, using E4 MAb. Ig+ cells were also demonstrated in formalin-fixed paraffin embedded tissue sections of spleen, kidney, thymus and smears of blood mononuclear cells in indirect immunoperoxidase test. The developed MAb was employed to detect pathogen-specific immunoglobulins in the sera of *C. batrachus* immunized with killed *Edwardsiella tarda*, by an indirect ELISA. This monoclonal antibody can be useful tool in immunological research and assays.

1. Introduction

Teleosts are the earliest evolutionary class to possess adaptive immunity which is composed of humoral and cell-mediated immunity. The adaptive immune responses are mediated by actions of 2 major groups of lymphocytes classified as B and T cells. Immunoglobulins (Ig), secreted by B cells, are the major effector molecules in humoral immunity and are directed to neutralize the pathogen or tag them for removal by the immune system. Teleost B cells produce 3 different immunoglobulin isotypes; namely, IgM, IgD and IgT. The IgM is the principal player in systemic immunity and IgT appears to be the teleost Ig class specialized in mucosal immune responses (Salinas et al., 2011). Monoclonal antibodies (MAbs) specifically reacting with Ig have proven to be a powerful tool for determining the level of total and specific Ig (Tang et al., 2010; Zhan et al., 2009) and number of Ig-positive (Ig+) cells in different tissues in healthy (Li et al., 2007) infected and vaccinated fish (Tang et al., 2010; Xu et al., 2011). Such monoclonal antibodies have also been used for immunolocalization of Ig+ cells in lymphoid organs (Sood et al., 2011; Tokuda et al., 2000) and have contributed greatly to improved understanding of the architecture and functioning of the fish immune system.

The fish *Clarias batrachus*, locally known as magur, is native to Southeast Asia. The natural distribution range of the species includes India, Bangladesh, Sri Lanka, Pakistan, Myanmar, Malaysia, Singapore, Philippines, Borneo, Java and Thailand (Talwar and Jhingran, 1992). The fish can occur in all types of freshwater but are more abundant in derelict and swampy waters with high turbidity. *C. batrachus* is a popular and valuable food fish owing to its good taste and low fat content, and fetches a high market price. Due to aerial respiration, the fish can be traded and sold live, thereby, ensuring a fresh food product. This catfish is at increased risk of developing infections by virtue of its preferential habitat in bottom zones of swampy waters, where the bacterial population may be 10–20 times higher than in water column (Lewis and Bender, 1961). There are reports of occurrence of diseases and health related problems in this species (Anonymous, 1981; Kanchanakhan, 2009).

MAbs have been raised to Ig of a number of teleost fish (Li et al., 2007) and most of the MAbs recognize the heavy chain of Ig (Scapigliati et al.,

Abbreviations: APES, 3-aminopropyltriethoxysilane; BSA, bovine serum albumin; c-ELISA, competitive ELISA; Cb-Ig, *Clarias batrachus* immunoglobulins; DAB, 3-3′ diaminobenzidine; DMEM, Dulbecco's Modified Eagle Medium; FITC, fluorescein isothiocyanate; FSC, forward scatter; HAT, hypoxanthine–aminopterin–thymidine medium; HC, heavy chain; Ig, immunoglobulins; IIPT, indirect immunoperoxidase test; LC, light chain; MAbs, monoclonal antibodies; MNC, mononuclear cells; MW, molecular weight; OD, optical density; OPD, ortho-phenylenediamine; PBS-T, phosphate buffer saline with 0.05% Tween-20; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, side scatter; TMB, 3,3′,5,5′-tetramethyl benzidine.

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1999). There is a report on purification and characterization of serum immunoglobulins of *C. batrachus* (Swain et al., 2004). However, there is no published information regarding reactivity of monoclonal antibodies in lymphoid organs of *C. batrachus*. In this paper, we describe the purification of serum immunoglobulins of *C. batrachus* (Cb-Ig), production of monoclonal antibodies to the purified immunoglobulins and their characterization. It is envisaged that monoclonal antibodies against serum Ig of *C. batrachus* will help in better understanding of immune system of this species.

2. Materials and methods

2.1. Fish immune sera preparation

Fifteen apparently healthy *Clarias batrachus*, weighing 100–150 g were acclimatized in fiber-reinforced plastic (FRP) tanks and divided in two groups (test and control). The test group comprised of 10 fish, whereas, five fish served as control. The fish in test group were immunized intra-peritoneally with 100 μg of bovine serum albumin (BSA) emulsified with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, USA) and subsequently boosted thrice with similar emulsion in Freund's incomplete adjuvant (Sigma-Aldrich) at 2 week intervals. The fish in control group were injected with phosphate buffer saline (PBS) emulsified in Freund's complete and incomplete adjuvant, similarly. Pre- and post-immunization blood samples (7 days after last injection) were collected from the fish and allowed to clot overnight at 4 °C. It was centrifuged at 2000 rpm to collect the serum. The harvested serum was stored at −20 °C.

Indirect hemagglutination (IHA) test was used to assess the humoral immune response to BSA in immunized fishes (Cho et al., 1976). Briefly, sheep erythrocytes (S-RBCs) were fixed in glutaraldehyde and sensitized with BSA. For the test, two-fold dilution of individual fish serum (1:2 to 1:512) was prepared in PBS in a 96 well microtiter plate, except RBC control wells. Equal volume of sensitized S-RBCs (50 μl) was added to each well, incubated for half an hour (h) and the highest dilution of serum showing agglutination was considered as IHA titer.

2.2. Purification of Ig by affinity chromatography

BSA-CL agarose column (Genei, India) was used to purify anti-BSA Ig from immunized fish following Rathore et al. (2008). SDS-PAGE was carried out under reducing and non-reducing conditions to check the purity and molecular weight (MW) of the purified Ig. Reduced samples were analyzed on 12% gel (Laemmli, 1970) while non reduced samples were analyzed on 3–12% polyacrylamide gel (Walker, 1996). The gels were subsequently stained with Coomassie Brilliant Blue to detect the polypeptides. The molecular weight of the bands in the gel was determined by LabWorks Image Acquisition and Analysis software (UVP BioImaging Systems) using appropriate MW markers.

2.3. Fish serum collection

Blood was collected from caudal vein of *C. batrachus* (100–150 g), *Clarias gariepinus* (400–500 g), *Heteropneustes fossilis* (80–100 g), *Channa striata* (200–250 g) and *Labeo rohita* (250–300 g) and allowed to clot. The serum was separated and stored at −20 °C. Serum from three individuals of each species was pooled for use in Western blotting and competitive ELISA.

2.4. Production of monoclonal antibodies

Monoclonal antibodies to Cb-Ig were raised following the standard procedure (Hamilton and Davis, 1995). Female BALB/c mice were procured from the Central Drug Research Institute, Lucknow and 2 mice were immunized with 50 μg of Cb-Ig emulsified in Freund's complete adjuvant (Sigma-Aldrich) by subcutaneous route. These mice were boosted twice with similar emulsion in Freund's incomplete adjuvant at 2 week intervals. Test bleeding of the mice was done on day 36 for testing the antibody titer. A final injection of Cb-Ig in PBS (25 μg) was given by intraperitoneal route to the mouse with higher antibody titer. Four days after last injection, the spleen cells from the mouse were collected and fused with myeloma cells (SP2/0) at a ratio of 10:1, using PEG–DMSO (Sigma-Aldrich). The fused cells were seeded in 96 well plates and grown in Dulbecco's Modified Eagle Medium (DMEM) containing hypoxanthine–aminopterin–thymidine (HAT, Sigma). The plates were checked for growth of hybridomas and positive clones were screened using indirect ELISA. The positive clones were subjected to single cell cloning and subcloning using limiting dilution method. The single clones were again checked by indirect ELISA and positive clones were further expanded. The class of monoclonal antibodies was determined by a mouse monoclonal antibody isotyping kit (Sigma-Aldrich).

An indirect ELISA was carried out for titration of mice sera and screening of wells containing hybridomas, for anti-Cb-Ig antibodies (Sood et al., 2011). The serum dilution giving 5 times OD to that of 0-day serum was considered as the titer. Similarly, the culture supernatants that gave 5 times OD to that of 0-day mouse serum on two occasions were selected for limiting dilution.

One of the strongly reacting MAbs (E4 MAb) was characterized by a number of immunological assays; (1) Western blotting to know the reactivity of anti-Cb-Ig MAb, (2) competitive ELISA (c-ELISA) to know the antigenic relatedness of Cb-Ig with whole serum of heterologous fish species, (3) flow cytometry to quantify Ig-positive (Ig+) cells in blood and lymphoid organs, (4) indirect immunoperoxidase test (IIPT) to demonstrate the reactivity of selected MAb in tissue sections of lymphoid organs and smears of blood mononuclear cells (MNCs) and (5) indirect ELISA to detect *Edwardsiella tarda*-specific antibodies in serum of immunized magur.

2.5. Western blot analysis

The reactivity of selected monoclonal antibody against reduced Cb-Ig and serum was checked using standard technique for Western blotting (Towbin et al., 1979). Briefly, after SDS-PAGE of reduced Cb-Ig and pooled sera samples of *C. batrachus*, *C. gariepinus*, *H. fossilis*, *C. striata* and *L. rohita*, proteins were electrophoretically transferred from unstained 12% gel to nitrocellulose membrane (Sigma-Aldrich) at 25 V for 2 h. After blocking in PBS with 5% skimmed milk powder, the nitrocellulose strips were incubated with 1:20 dilution of culture supernatant of E4 MAb. Following three washings, the strips were incubated with 1:4000 dilution of rabbit anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) and the reaction was visualized using 3,3′,5,5′-tetramethyl benzidine (TMB) substrate solution (Sigma-Aldrich).

Immunoblotting analysis was also carried out following SDS-PAGE of non-reduced Cb-Ig on a gradient (3–12%) polyacrylamide gel.

2.6. Competitive ELISA

The optimal dilution of E4 MAb giving an OD in the range of 1.0 ± 0.2 was determined by an indirect ELISA, using doubling dilutions of MAb on Cb-Ig coated plates. ELISA plates were coated with 50 μl well^{-1} of Cb-Ig (1 μg ml^{-1}) at 37 °C for 1 h. Serial dilutions (1:200 to 1:25,600) of sera samples of *C. batrachus*, *C. gariepinus*, *H. fossilis*, *L. rohita* and *C. striata* were used as competitor. The competition was carried out by adding 50μl well^{-1} of serially diluted competitor along with 50 μl of optimally diluted E4 MAb, in duplicate. In control wells, MAb was added without any competitor. The plate was incubated overnight at 4 °C and subsequently washed. The plate was again incubated with 50 μl of goat anti-mouse IgG peroxidase conjugate (1:4000) at 37 °C for 1 h. After washing, color reaction was developed by adding OPD. The