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Production and characterization of monoclonal antibodies against tilapia *Oreochromis niloticus* immunoglobulin

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Abstract

Eight monoclonal antibodies (MAbs) against tilapia immunoglobulins (Ig) were produced and characterized. All of the MAbs are functional for use in enzyme-linked immunosorbent assays (ELISAs) and immunoblotting applications. Characterization of the MAbs for mouse Ig class and subclass indicate that four were IgG 2b, two were IgG 2a, one was IgG 3, and one was IgM K class. Western blot analysis of the eight hybridomas with purified Nile tilapia Ig indicated that all eight MAbs were heavy chain specific and further analysis indicated that all eight MAbs react with tilapia Ig H chain and cross-reactive with other tilapia species and their hybrids.

Keywords: Tilapia; Immunoglobulin; Monoclonal antibodies; Isotypes

1. Introduction

Monoclonal antibodies (MAbs) technology provides the ability to enhance immunological or serological analysis by providing a uniform reagent of potentially unlimited quantity. MAb specifically reacting with immunoglobulin (Ig) provides a powerful tool

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for monitoring antibody production following infection or vaccination, and for immunoblotting analysis of antigens recognized by the host immune system. During the last decade, MAbs have been produced against serum Ig of a variety of marine or freshwater fish, including carp (*Cyprinus carpio*) (Secombes et al., 1983), rainbow trout (*Oncorhynchus mykiss*) (De Luca et al., 1983; Thuvander et al., 1990; Sanchez et al., 1993), channel catfish (*Ictalurus punctatus*) (Lobb and Clem, 1982), Atlantic salmon (*Salmo salar*) (Pettersen et al., 1995; Magnadottir et al., 1996), sea bream (*Sparus aurata*) (Navarro et al., 1993), sea bass (*Dicentrachus labrax*) (Romestand et al., 1995 ; Scapigliati et al., 1996; Dos Santos et al., 1997), turbot (*Scophthalmus maximus*) (Estevez et al., 1994), European eel (*Anguilla anguilla*) (Van der Heijden et al., 1995), goldfish (*Carassius auratus*) (Siwicki et al., 1994), and red drum (*Sciaenops ocellatus*) (Macdougal et al., 1995). Our literature review further indicated that MAbs have not been developed against the Ig of any tilapia species, despite the fact that tilapia represents one of the most widely produced food fish in the world (Stickney,1986).

The lack of reagents to study the tilapia immune response is partially due to the lack of significant pathogens in cultured tilapia. Recently, however, with expansion of recirculating culture technology for tilapia, *Streptococcus* sp. as pathogen have emerged, particularly *S iniae* (Kitao et al., 1981; Miyazaki et al., 1984; Al-Harbi, 1994; Eldar et al., 1994; Perera et al., 1994).

This study was initiated to produce MAbs to tilapia serum Ig that could be used to monitor humoral antibody in tilapia following natural infections or vaccination and to evaluate antigen recognition by immunoblotting. This study reports the characterisation of the MAbs produced, and evaluates their application in other tilapia species and their hybrids.

2. Materials and methods

2.1. Experimental fish and sampling

Adult Nile tilapia *Oreochromis niloticus*, Blue tilapia *O. aureus*, Mississippi Commercial hybrid *O. aureus* × *O. niloticus* × *O. mossambicus*, and Florida Red hybrid *O. urolepis hornorum* × *O. mossambicus*, weighing 200–300 g, were obtained from a local fish farm. Blood was drawn from the caudal vein, and allowed to clot at room temperature for 1 h and stored overnight at 4°C. Serum was separated the next day by centrifugation at $2000 \times g$ for 10 min and then stored in 0.5-ml aliquots at -20°C until use.

2.2. Purification of tilapia immunoglobulin

For primary mouse immunizations, Nile tilapia Ig was partially purified from serum by precipitation with 50% saturated ammonium sulphate. The precipitate was pelleted by centrifugation at $15{,}000 \times g$ for 20 min, re-suspended in phosphate buffered saline (PBS, pH 7.2), and dialysed extensively against PBS. The dialysed solution was clarified by centrifugation at $15{,}000 \times g$ for 20 min and stored at -70°C.

For evaluation of the MAbs for ELISA reactivity and immunoblotting capability, tilapia, Ig was purified by a mannan-binding protein (MBP) affinity column following the manufacturer's protocol (ImmunoPure R IgM purification kit, Pierce Chemical, Rockford, IL, USA). Briefly, the column was washed with 5 ml of the immuno Pure MBP preparation buffer at room temperature and then equilibrated with 20 ml of immune Pure R IgM binding buffer at 4°C at a flow rate of 1 ml/min. Fish serum was dialyzed overnight at 4°C against Tris-buffered saline (TBS; 20 mM Tris, 1.25 M sodium chloride, 0.02% sodium azide buffer, pH 7.4). After dialysis, the serum was diluted 1:1 with the immune Pure R IgM binding buffer, and 1 ml of the diluted serum was applied to the column which was then incubated at 4°C for 30 min. After incubation, the column was washed with 42 ml of binding buffer to remove unbound protein and 3 ml of immune Pure R IgM elution buffer was added and allowed to incubate for 1 h at room temperature. The column was then eluted with 45 ml of elution buffer and 3 ml fractions were collected. Protein content of all fractions was measured by absorbance at 280 nm and fractions with absorbances > 0.02 were concentrated by centrifugation in a Centricon-10 micro concentrator (Amico, Danvers, MA, USA). Concentrated fractions were stored at -70°C until use.

Total protein concentrations of the various serum samples and serum fractions were quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), which is based on a dye-binding procedure (Bradford, 1976). The assay was conducted according to the manufacturer's protocol with bovine serum albumin as the protein standard. The protein content of the MBP column fractions was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), using a Mini-protean II electrophoresis unit (Bio-Rad). After electrophoresis, the gels were stained for 2 h with Coomassie brilliant blue R-250 (Bio-Rad). Gel images were recorded on a Flour-S MultiImager and analyzed using quantity one quantitation Software (Bio-Rad).

2.3. Production of monoclonal antibodies

Balb/C mice were immunized by intraperitoneally with 45 μg of ammonium sulphate precipitated Nile tilapia Ig fraction emulsified with an equal volume of Freunds complete adjuvant (FCA). After 4 and 9 weeks, mice were given booster doses of 76.5 μg of the MBP-purified Nile tilapia Ig without adjuvant. At 4 days after the second booster, spleen cells were collected from the mouse and fused with myeloma cell line, SP 2/0-Ag-14 (Boehringer Mannheim, Indianapolis, IN) at a ratio of 4:1, using 50% polyethylene glycol 1500 . The cells were distributed into 96-well tissue culture plates in HA medium (10^5 cells per well in 100 μl), and the culture medium was changed every 3–5 days. After 12–14 days, the supernatant from those wells growing hybridomas were screened by indirect enzyme-linked immunosorbent assay (ELISA), for the production of anti-tilapia Ig antibodies. Positive wells were expanded, sub-cloned, and evaluated for anti-tilapia Ig activity using ELISA and Western Blot as described below. MAb class and subclass were determined by an ELISA-based mouse Ig Isotyping Kit (ISO-1, Sigma, St. Louis, MO, USA) and following the instruction of the manufacturer.

2.4. ELISA

Hybridomas were screened for anti-tilapia Ig activity by ELISA. Briefly, Dynatech Immulon flat bottom microtitre plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with tilapia Ig by adding 100 μl/well of carbonate buffer (CB: 32 mM Na_2CO_3, and 68 mM $NaHCO_3$) containing 1 μg/100 μl of MBP-purified tilapia Ig and incubated overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween-20 (TPBS), and blocked for 2 h at room temperature with 200 μl of 3% bovine serum albumin (Boehringer Mannheim) in CB. After washing three times with TPBS, 50-μl samples of hybridoma culture supernatants were added and incubated for 1 h at room temperature. Plates were washed five times with TPBS and incubated for 1 h at room temperature with 100 μl/well of affinity-purified goat-anti-mouse Ig–horseradish peroxidase conjugate (GAM–HRP; Bio-Rad, Richmond, CA, USA) diluted 1:2000 in CB. After washing five times with TPBS 125 μl of the developing solution, 0.035% (w/v) of *O*-phenylenediamine dihydrochloide (Sigma) and 0.02% (v/v) hydrogen peroxide prepared in 0.15 M citrate–phosphate buffer, pH 5.0,was pipetted into each well. After 10 min of colour development in darkness, the reaction was stopped with 100 μl per well of 2% (v/v) oxalic acid. Optical density was measured at 492 nm on a Dynatech MR-500 ELISA plate reader (Dynatech Laboratories).

2.5. Western blotting

After SDS-PAGE, proteins were electrophoretically transferred from unstained gels to 0.45 μm nitrocellulose membranes at 100 V constant for 1 h in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3. The membranes were then dried and stored in the dark until immunodetection. Proteins blotted to nitrocellulose were probed with supernatant from hybridomas with anti-tilapia Ig activity demonstrated by ELISA. Thin strips of the membranes were immersed in Tris buffered saline (20 mM Tris, 137 mM NaCl, pH 6.7 with 0.1%Tween-20) (TBST) and 5% non-fat dry milk to block non-specific binding to the membrane. Following five washes in TBST, strips were immersed in the supernatants, either undiluted or diluted 1:4 in TBST for 1 h at room temperature. Probed strips were again washed five times in TBST and immersed in GAM–HRP (Bio-Rad) diluted 1:4000 in TBST for 1 h at room temperature. Positive bands were detected using enhanced chemiluminescence (Amersham International, Buckinghamshire, England, UK) and blue-light sensitive autoradiography film (Amersham). Strips with positive bands were compared with unprobed strips stained with colloidal gold total protein stain (Bio-Rad) to confirm the identification of bands as heavy or light chain specific. Strips incubated with TBST without the MAbs or GAM–AP were also used as controls. Broad range SDS-PAGE standards (Bio-Rad) were used for the estimation of molecular weights.

3. Results

The MBP affinity column (Pierce Chemical) successfully isolated serum Ig from tilapia. Serially eluted 3 ml fractions numbered 1–4 demonstrated significant protein

Table 1
Characterisation of MAbs specific for tilapia Ig

MAbs no.	Isotype	ELISA	Immunoblot	Chain specificity
81-11	IgM	+	+	H
149-1	IgG 2b	+	+	H
156-6	IgG 2a	+	+	H
455-5	IgG 2a	+	+	H
455-18	IgG 3	+	+	H
455-19	IgG 2b	+	+	H
455-26	IgG 2b	+	+	H
466-25	IgG 2b	+	+	H

content with optical densities greater than 0.02 at 280 nm. Analysis of the fractions by SDS-PAGE revealed bands corresponding to the heavy and light chains of tilapia Ig as described by Smith et al. (1993).

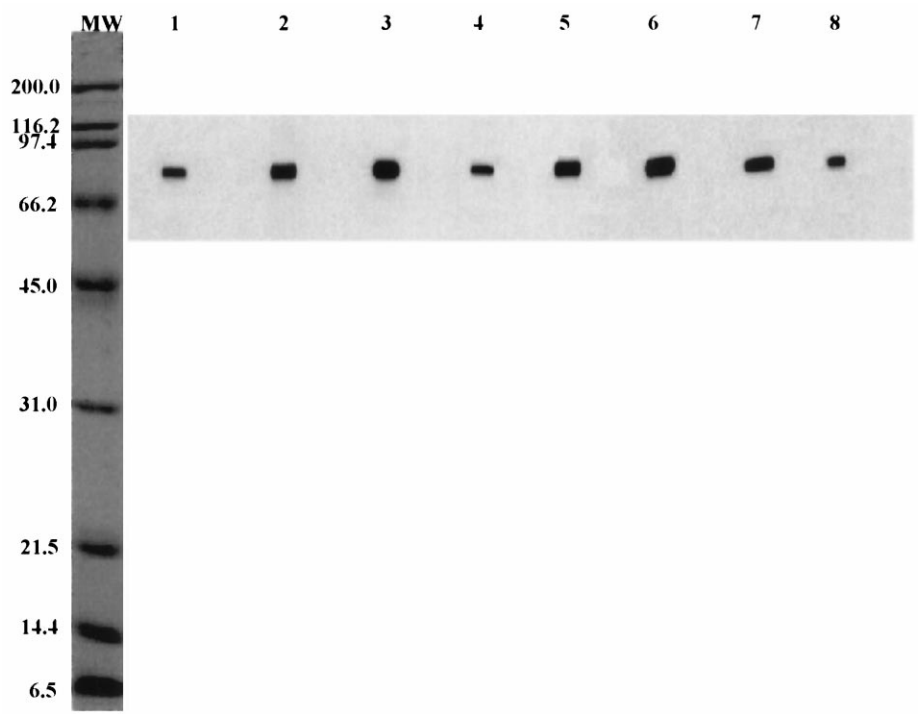

Fig. 1. Western blot of reduced MBP-purified Nile tilapia immunoglobulin. Immunostained with various MAbs. Lane 1: immunoreaction with MAb 81-11; Lane 2: immunoreaction with MAb 149-1; Lane 3: immunoreaction with MAb 156-6; Lane 4: immunoreaction with MAb 455-5; Lane 5: immunoreaction with MAb 455-18; Lane 6: immunoreaction with MAb 455-19; Lane 7: immunoreaction with MAb 455-26; Lane 8: immunoreaction with MAb 466-25; and high molecular weights standards with their respective molecular weight (kDa).

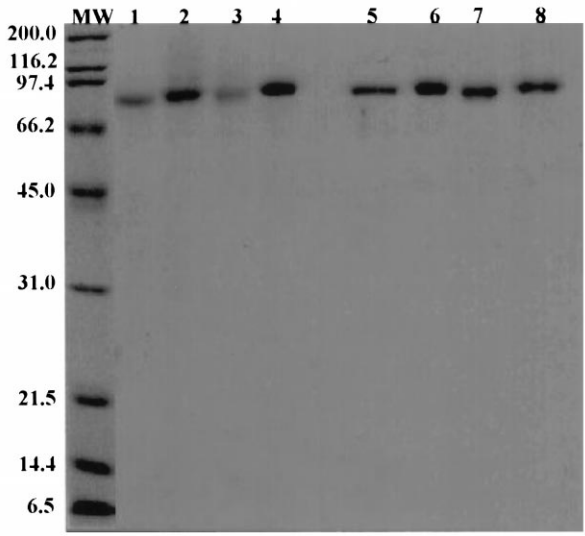

Fig. 2. Western blot of reduced tilapia whole serum and MBP-purified tilapia immunoglobulin immunostained with MAb (455-19). Lane 1: *O. niloticus* whole serum; Lane 2: *O. aureus* whole serum; Lane 3: *O. aureus* × *O. niloticus* × *O. mossambicus*, whole serum; Lane 4: *O. urolepis hornorum* × *O. mossambicus*, whole serum; Lane 5: purified *O. niloticus* IgM; Lane 6: purified *O. aureus* IgM; Lane 7: purified *O. aureus* × *O. niloticus* × *O. mossambicus* IgM; Lane 8: purified *O. urolepis hornorum* × *O. mossambicus* IgM; and high molecular weights standards with their respective molecular weight (kDa).

Initial ELISA screening of the spleen cell/hybridoma fusions demonstrated 121 wells that reacted with tilapia serum. Subsequent ELISA screening using MBP-purified tilapia Ig yielded 46 clones that were selected, sub-cloned and re-tested by ELISA. After sub-cloning, eight clones were stabilized and selected for further characterization on the basis of their strong reactivity. Results of the characterization are summarized in Table 1. Briefly, the class and subclass characterization of the Igs produced by these hybridoma cell lines indicated that four were IgG 2b, two were IgG 2a, one was IgG 3, and one was 1 gM/K class. Western blot analysis of the eight hybridomas, with the MBP-purified Nile tilapia Ig indicated that all eight MAbs were heavy chain specific (Fig. 1). Additional Western blot analysis with MBP-purified Ig from three other species/hybrids indicated that all eight MAbs recognized the heavy chain of all three tilapia species, although there was some minor variation in the molecular weights of the chains. Fig. 2 presents a representative blot of heavy chain detection for both MBP-purified tilapia Ig and whole serum of all of the tilapia evaluated.

4. Discussion

This study reports the first production of MAbs specifically directed against tilapia Ig. A panel of eight MAbs specifically directed against *O. niloticus* Igs was demonstrated to be useful in both ELISA and Western blotting applications. In addition, all

eight MAbs recognized and showed strong reactivity with IgM from *O. aureus* and two different hybrids *O. aureus* × *O. niloticus* × *O. mossambicus*, and *O. urolepis hornorum* × *O. mossambicus*. Homology between Igs of fish from the same genus (as evidenced by MAbs binding) has been reported previously for salmonids (Thuvander et al., 1990; Sanchez et al., 1993), turbot (Estevez et al., 1994), and goldfish (Siwicki et al., 1994). Interestingly, the heavy chain of all species of tilapia appeared to be slightly different from each other in size. This size variation is most likely due to variation in the degree of glycosylation of the heavy chain, and was also evident in the initial description of tilapia Ig (Smith et al., 1993).

The MBP affinity column provided an easy, one-step method for partial purification of tilapia Ig. Although inadequate quantity and purity was achieved for comprehensive analysis, the power of hybridoma cloning enabled the isolation of MAbs specific for tilapia Ig heavy chain. The utility of MBP for IgM purification is related to binding of carbohydrate moieties of the Ig, similar to C1q binding, and is involved in the initiation of the complement cascade (Ohta et al., 1990). Previous evaluation of the MBP system for fish Ig purification indicated species variability in efficiency, possibly related to relative glycosylation of the Ig. For example, documentation provided by the manufacturer indicates that Ig represented 24.2% of rainbow trout serum applied to the column and 94.7% of the collected fraction, while for lake trout the numbers were 5.3% and 73.1%, respectively (Wendelborn et al. 1992).

In conclusion, all of the MAbs produced in this study were useful in ELISA and immunoblotting procedures and should prove to be useful tools in infection and vaccination studies on tilapia species. These reagents can be used to follow both Ig responses and antigen recognition following infection and or vaccination. In addition, previous exposure to tilapia infectious agents can be evaluated.

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