

## Characterization of Carp (*Cyprinus carpio* L.) Immunoglobulin Structure

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**ABSTRACT** : Serum immunoglobulins (Igs) from Israeli carp were purified using affinity chromatography. Fish were immunized with purified mouse IgG, and the specific fish antibodies were purified from the immune serum on a mouse IgG-immobilized agarose gel. Rabbit anti-Israeli carp Igs (R  $\alpha$  I. carp Igs) antibodies were produced following hyperimmunization with mouse IgG specific carp antibodies. SDS-PAGE analysis under reducing condition showed that Israeli carp Igs were composed of two  $\mu$ -like heavy chains with about 82 and 50 kd, respectively, and one light chain with about 25 kd. On immunoblotting analysis, however, R  $\alpha$  I. carp Igs failed to react with the light chain. When both protein A and protein G-purified normal carp Ig were compared with mouse IgG-specific Israeli carp Ig, no significant structural differences among them were observed. To investigate if there is any homology between other fish Ig molecules, cross-reactivity of R  $\alpha$  I. carp Igs against Ig molecules from 6 different fish sera and mouse control serum was checked on immunoblotting analysis. As a result, R  $\alpha$  I. carp Igs responded to Israeli carp, common carp, and tilapia Ig molecules. In flow cytometry study, however, R  $\alpha$  I. carp Igs appeared to recognize 42.0%, 35.8% and <5% of Israeli carp, common carp and tilapia Ig<sup>-</sup> head kidney cells, respectively. The result suggests the heterogeneity between receptor Igs on B-like lymphocytes and soluble Igs in serum. It is crucial to obtain pure fish Igs to produce reagent antibodies as tools for the study on their specific immune responses. (*Asian-Aust. J. Anim. Sci.* 2002, Vol 15, No. 2 : 290-296)

**Key Words** : Affinity Chromatography, Flow Cytometry, Carp Ig, Tilapia

### INTRODUCTION

Fish are a heterogeneous group of vertebrates that include hagfish, lampreys, cartilaginous fish, and bony fish, all of which are capable of mounting antibody responses (Warr and Marchalonis, 1982). In past years, much has been learned about the antibodies and the genes encoding them in the elasmobranchs and the teleosts. Information in the case of the hagfish and lampreys is still rather incomplete and difficult to interpret, in that the definitive characterization of the molecules in these species, described as antibodies or immunoglobulins (Igs) (Marchalonis and Edelman, 1968; Raison et al., 1978; Hanley et al., 1990; Varner et al., 1991) remains to be completed. The predominant Ig in the blood of teleosts and most sharks is an IgM-like molecule. It consists of equimolar amounts of heavy ( $\mu$ ) and light (L) chains arranged in a basic unit containing 2  $\mu$  and 2 L chains. IgM exists either in polymeric forms, a tetramer ( $(\mu_2L_2)_4$  in teleosts, and pentamer ( $(\mu_2L_2)_5$  in sharks, or in monomeric forms ( $\mu_2L_2$ ) in sharks and some teleosts (Warr and Marchalonis, 1982). In the teleosts, where the antibody response has been studied quite thoroughly, there is no reason to think that these low molecular weight Igs are either the structural or functional equivalents of mammalian IgG.

The structure and function of fish IgM is of special interest, this being the first Ig to appear in evolution and the

only Ig class of lower vertebrates. It has been demonstrated that there is a significant homology between the amino acid sequence of IgM $\mu$  chain from very diverse species (Bengtén et al., 1991; Andersson and Matsunaga, 1993; Lee et al., 1993). Bournaud et al. (1995) reported the purification of an IgM-like Ig from sea bass, but no characterization of the molecule was provided except its native molecular weight. As for the family Sparidae, a few complete studies are available in the literature (Lobb and Clem, 1981). Thus far, most teleostean serum Igs have been characterized as tetrameric IgM-like macroglobulins with molecular weights between 600 kd and 800 kd (Acton et al., 1971; Litman, 1976; Dorson, 1981). However, heterogeneity has been reported for the heavy and light chains from some fish species (Kobayashi et al., 1982; Lobb et al., 1984; Lobb and Olson, 1988; Sanchez et al., 1989) as well as the presence of different covalently linked IgM forms (Glynn and Pulsford, 1990; Clem and McLean, 1975). In addition, low molecular weight Igs have been described (Clem and McLean, 1975; Warr, 1983). Affinity chromatography techniques have previously been used for the purification of fish antibodies. Among them, variable results have been obtained with protein A-based methods depending on the particular fish species (Suzuki et al., 1990; Estevez et al., 1993a; Estevez et al., 1993b). These techniques are improved when a specific interaction of antigen-antibody molecules is involved. The molecule attached to the insoluble support matrix may be an antibody or an antigen against which the fish antibody has been produced (Kofod et al., 1994).

In the present study, carp antibodies were produced

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against mouse IgG and purified by mouse IgG-coupled Sepharose 4B, protein-A or G affinity column chromatography. The structural properties of carp Igs were investigated on the basis of electrophoretic analysis. Furthermore, to compare structural homologies between Igs in carp serum and those in other different fish species sera, we used rabbit antibodies against carp Igs. In addition, a flow cytometry analysis was performed to study whether B-like lymphocytes with surface Ig receptors are present in head kidneys from various kinds of fishes as well as carp.

The purpose of the present work was to characterize serum Igs of Israeli carp for the study of their specific immune response.

## MATERIALS AND METHODS

### Production of fish immune sera

Five adult two-year-old *Cyprinus carpio* L. (mean weight 500-600 g) were maintained in tanks under controlled temperature (18-20°C). Fish were fed a standard commercial diet and were acclimatized to experimental conditions for at least two weeks before immunization. Fish were injected intraperitoneally on day zero with 200 µg of purified mouse IgG (Sigma, U.S.A.) per fish, in 0.2 ml of a 1:1 emulsion in Freund's complete adjuvant (FCA). Boosters were injected on days 7, 14 and 21 with the same doses of mouse IgG in Freund's incomplete adjuvant (FIA). Final booster was challenged on days 28 with the same doses of mouse IgG in 0.2 ml of sterile Hank's balanced salt solution (HBSS). Following each boosting injection, serum samples were harvested for their serological study. Control fish received 0.2 ml of HBSS in each injection. Both immunized and control fish were anaesthetized with MS-222 (Sigma) on day 40, and bled to death from the caudal vein, using non-heparinized syringes. The blood was allowed to clot at 20°C and then at 4°C.

### Purification of fish Ig

Fish anti-mouse IgG antibodies were purified by affinity chromatography as described by Smith et al. (1993). Briefly, 2 ml each of mouse IgG, protein A, or protein G-coupled agarose-beads (Sigma) were gravity-packed on a 10×0.5 cm syringe column, respectively, washed with 200 ml of 0.01 M phosphate buffered saline, pH 7.2 (PBS) containing 0.5 M sodium chloride (equilibration buffer) and then stripped with 20 ml of 0.1 M glycine-NaOH, pH 11 (elution buffer). After re-equilibrating the column, 2 ml of pooled immune fish sera were diluted 1:1 with equilibration buffer, 0.22 µm-filtered and applied to the column. The column was washed again with equilibration buffer, until the baseline was restored. Bound proteins were then eluted with 5-10 ml of elution buffer, and collected in one fraction over 1 ml of 0.1 M Tris-HCl, pH 7.2 (neutralization buffer). The

proteins were concentrated and changed to a physiological environment (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 0.01% thimerosal) by diafiltration (Centriprep-100 concentrators, Amicon, Beverly, MA, U.S.A.) following manufacturer instructions. Protein content of the concentrates was determined by the method of Bradford (1976) (Protein Assay kit, BioRad), sterile-filtered and stored at 4°C until used.

### Rabbit antisera preparation

New Zealand rabbits were subcutaneously injected on day zero with 250-300 µg of affinity-purified fish Igs emulsified 1:1 in FCA, in a total volume of 1 ml per rabbit. Animals were boosted in the same way on days 14 and 34 with the antigens prepared in FIA, and on day 45 without adjuvant. Blood samples were taken on days zero (preimmune serum), 41 and 52 by ear vein puncture, and the sera prepared as described above. The titer was determined by ELISA according to the method of Palenzuela et al. (1996).

### SDS-PAGE

To identify whether fish Igs were efficiently purified by affinity chromatography, 10% denatured polyacrylamide gel electrophoresis was performed according to the Laemmli (1970) method.

### Immunoblot analysis

The structural properties of fish Igs were investigated by immunoblot analysis. Total fish serum proteins and affinity-purified Igs were reduced and denatured by 5-6 min boiling in loading buffer (62 mM Tris-HCl, pH 6.8, containing 2% [w/v] SDS, 10% [v/v] glycerol, 0.05% [w/v] bromophenol blue and 0.1 M dithiothreitol) and electrophoresed on preparative 10% polyacrylamide gels, and electrotransferred to 0.45 µm nitrocellulose membranes (BioRad) for 1 h at 250 mA. Membranes were blocked for 1 h at room temperature (RT) in Tris-buffered saline (TBS) containing 5% skimmed milk, washed in TBS and mounted in a screening device (Miniprotean II multiscreen apparatus, BioRad). Serial twofold dilutions in TBS of the different rabbit antisera were applied to the channels and incubated for 3 h at RT. After washing, goat anti-rabbit IgG-AP conjugate diluted 1/3000 in TBS, pH 7.5, containing 0.05% (v/v) Tween-20 and 3% skimmed milk (S-TTBS) was delivered to the channels, incubated for 2 h at RT and washed. The membranes were rinsed again in TBS, and the presence of immune complexes detected by incubation in substrate (NBT&BCIP) (Promega, Madison, WI) for 15 min.

### Flow cytometry analysis

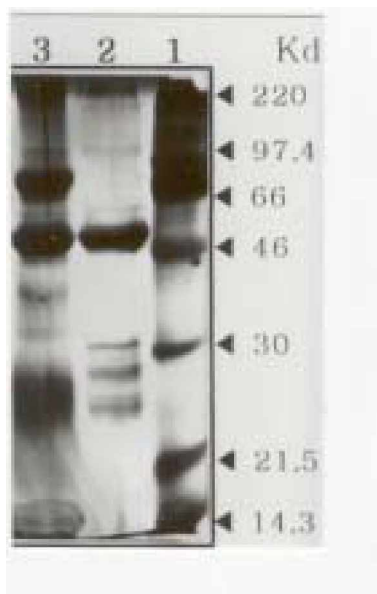
In order to study whether RαI, carp Igs recognize

surface Ig receptor on fish B-like cells. flow cytometry analysis was performed with fish head kidney and spleen cells. Aliquots of  $5 \times 10^5$  cells were labelled for 30 min on ice with RoI. carp Igs. After washing, cells were incubated for 30 min on ice with fluorescein-conjugated goat anti-rabbit IgG. The fluorescence of  $10^4$  viable cells was measured by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA).

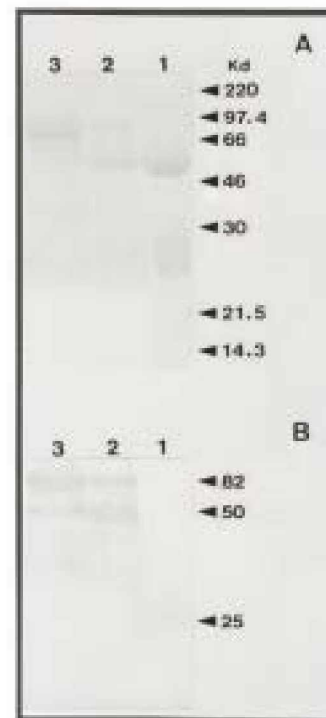
## RESULTS

### Two heavy chains of Israeli carp Igs

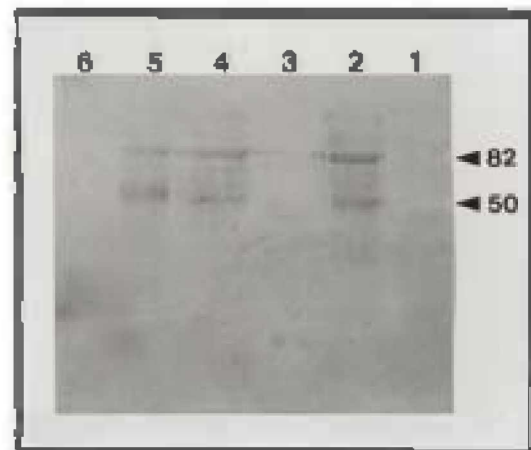
Israeli carp Igs purified by mouse IgG-, protein A-, or protein G-agarose affinity chromatography were electrophoresed. Israeli carp Igs were identified to be composed of two heavy chains (82 and 50 kd) and one light chain (25 kd) (figures 1 and 2A). The band pattern of Protein A-purified Igs was somewhat different from that of protein G-purified Igs, i.e., 50 kd band was clearly observed in the sample of protein G-purified Igs, but not in that of protein A-purified Igs (figure 2A). However, in the immunoblot analysis, 50 kd heavy chain band was identified (figure 2B), suggesting a possibility that protein A and G can recognize different fish Ig molecules, respectively. Figure 3 shows the result of immunoblot analysis for affinity-purified Israeli carp Igs. Two non-identical heavy chains (82 and 50 kd) were detected, whereas no light chains were observed in any three different samples. RoI. carp Igs did not cross-react with purified



**Figure 1.** SDS-PAGE for carp Igs purified by mouse IgG-agarose affinity chromatography. Lane 1, protein marker; 2, purified mouse IgG for control; 3, mouse IgG-specific carp Igs. Protein bands were silver stained



**Figure 2.** SDS-PAGE (A) and Immunoblot analysis (B) for affinity chromatography-purified carp Igs. Panel A, proteins bands stained with Coomassie blue; Panel B, goat anti-rabbit IgG-alkaline phosphatase-stained same protein samples used in the panel A. Lane 1, mouse IgG; 2, protein G-purified carp Igs; 3, protein A-purified carp Igs



**Figure 3.** Immunoblot analysis for affinity chromatography-purified carp and catfish Igs, and their sera. Lane 1, mouse IgG; 2, carp serum; 3, catfish serum; 4, protein G-purified carp Igs; 5, mouse IgG-agarose-purified carp Igs; 6, protein G-purified catfish Igs

mouse IgG or catfish Igs. In order to investigate whether the purified fish Igs were contaminated with other protein molecules or composed of two different intra-heavy chains,

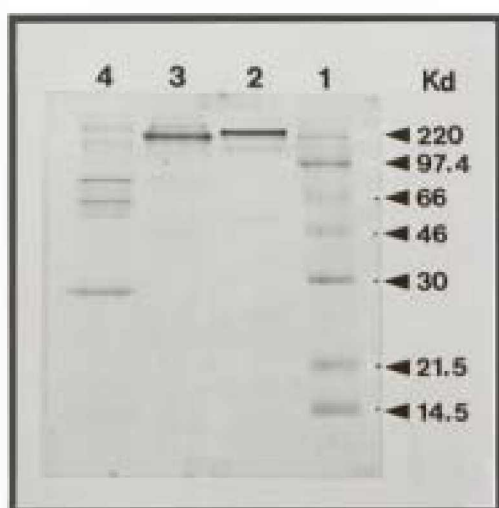
non-denatured SDS-PAGE was performed. As shown in figure 4, only one band appeared at over 220 kd, suggesting that purified fish I<sub>G</sub>s were not contaminated but constructed of two non-identical heavy chains. However, the possibility of the presence of two different I<sub>G</sub> molecules in fish serum was not excluded.

#### Homology between tilapia I<sub>G</sub>s and Israeli carp I<sub>G</sub>s

To investigate structural homologies among fish I<sub>G</sub>s, six different fish sera were electrophoresed followed by immunoblotting with RαI. carp I<sub>G</sub>s. As shown in figure 5A, serum proteins of Israeli carp and tilapia appeared similar to those of common carp. In immunoblot analysis, two heavy chains (82 and 50 kd) from Israeli carp, common carp and tilapia I<sub>G</sub>s were detected, but not from mouse I<sub>G</sub>s as well as other fish I<sub>G</sub>s (figure 5B). No light chains from the three fish I<sub>G</sub>s were observed.

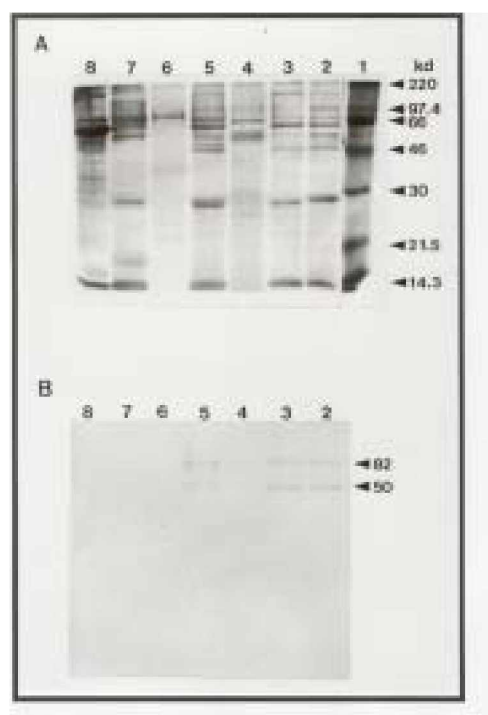
#### Alteration in I<sub>G</sub> structure following the time-based immunization

Two carp were periodically immunized with purified mouse IgG. Fish blood samples were collected following each time of antigen immunization. To identify how isotypes and protein concentration of carp I<sub>G</sub>s were altered after immunization of antigen, electrophoresis and immunoblot analysis were performed. As shown in figure 6A, the band patterns of serum proteins were observed with a slight difference, especially in lane 4, 4' and 5'. Figure 6B shows the result of immunoblot analysis on the same samples used in figure 6A. Unexpectedly, no change of I<sub>G</sub> isotype or concentration was detected following incubation of RαI. carp I<sub>G</sub>s with serum samples.



**Figure 4.** Native electrophoresis for purified fish I<sub>G</sub>. Lane 1, protein marker; 2, mouse IgG; 3, protein G-purified carp I<sub>G</sub>s; 4, carp serum. Protein samples were stained with Coomassie blue

#### Flow cytometry analysis

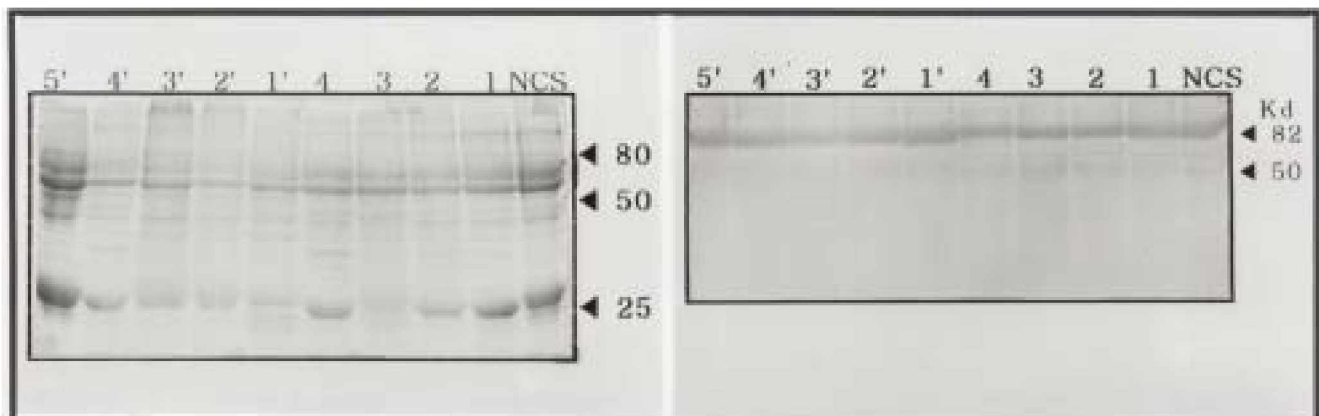


**Figure 5.** Comparison of I<sub>G</sub> band patterns in various kinds of fish sera by using rabbit anti-carp I<sub>G</sub> antibodies. Panel A, silver stained fish serum samples following SDS-PAGE; panel B, immunoblot analysis with same samples used in panel A by using rabbit anti-carp I<sub>G</sub>s. Lane 1, protein marker; 2, Israeli carp serum; 3, normal carp serum; 4, catfish serum; 5, tilapia serum; 6, rock fish serum; 7, flounder serum; 8, eel serum

In order to investigate whether serum I<sub>G</sub>s in fish are also expressed on fish B-like cell surfaces like mammalian, we labeled carp and other fish head kidney cells with RαI. carp I<sub>G</sub>s. Both common and Israeli carp kidney cells appeared I<sub>G</sub> receptor positive, whereas rabbit antibodies failed to react with tilapia cells, suggesting a structural disparity of epitopes present on serum I<sub>G</sub>s and cell surface I<sub>G</sub> receptor (table 1). As expected, no cross-reaction of rabbit antibodies was observed against other fish B-like cells (table 1).

#### DISCUSSION

The structure and function of IgM is of special interest, this being the first I<sub>G</sub> to appear in evolution and the only I<sub>G</sub> class of lower vertebrates. It is generally accepted that the major serum IgM of teleosts has a tetrameric form with a molecular weight (MW) between 600 and 900 Kd (Rombout et al., 1993; Navarro et al., 1993; Koumans-van Diepen et al., 1995; van der Heijden et al., 1995). The major plasma I<sub>G</sub> found in fish is a molecule that is generally called



**Figure 6.** The properties of carp IgG following time-based mouse IgG immunization. Serum samples were collected after each boosting time from two different carp immunized with mouse IgG. The number of 1 and 1', serum samples 7 days after primary immunization; 2 and 2', 7 days after 1st boosting (total 14 days); 3 and 3', 7 days after 2nd boosting (total 21 days); 4 and 4', 7 days after 3rd boosting (total 28 days); 5', 3 days after final boosting (total 33 days).

**Table 1.** Flow cytometry analysis for binding of rabbit anti-Israeli carp IgG to surface Ig receptors on fish B-like cells

Fishes	Cells	RαI. cIgs (%)*
I. Carp	K	42.0
	S	35.4
Carp	K	35.8
	S	26.4
Rainbow trout	K	0.5
	S	0
Catfish	K	1.0
	S	0.5
Tilapia	K	4.3
	S	4.8
Flounder	K	0
	S	1.3

\* Normal rabbit IgG was used as a negative control. The number of cells applied in the reaction was  $5 \times 10^5$ . GαRIgG-FITC was used for identifying Ig receptor positive cell population. K, kidney cells; S, spleen cells.

The results are representative of three experiments.

IgM. It consists of  $\sim 70,000$  relative molecular mass (Mr) heavy chains ( $\mu$ ) and  $\sim 22,000$ – $25,000$  Mr light chains (L) in equimolar amounts. IgM has been isolated (and at least partially characterized) from many species of fish, both chondrichthyan and osteichthyan, and much of the earlier work is summarized and referenced (Sigel et al., 1972). In the present study, we developed polyclonal rabbit antibodies against carp IgG which were purified by protein A affinity chromatography and characterized structural properties of fish IgG on a reduced SDS-PAGE. Differently as stated in references above, two heavy chains (82 and 50 kD) and one light chain (25 kD) were observed in carp IgG. The

result suggests that carp IgG are composed of two different heavy chains and one light chain. Antibodies are among the most structurally complex of biological molecules. This complexity transcends their final protein form and includes the intricate genetic mechanism required to produce the wide repertoire of antibodies. The need for such complexity is obvious, for within their structures must lie the ability to bind a virtual universe of pathogens and ensure their destruction and removal. The construction and function of such large ( $\sim 1,000$  kD) and complex molecules have yet to be fully delineated even for the well-studied murine and human systems. Therefore, more works should be performed to identify whether two different heavy chains are formed by inter-disulfide bonds in one Ig molecule or two non-identical isotypes of Ig are present in Israeli carp serum. In a native SDS-PAGE analysis, only one protein band was detected, indicating that the purified fish IgG were not contaminated by some other proteins but are internally constructed with two different heavy chains. Nonetheless, we still cannot exclude the possibilities of existence of two non-identical heavy chains in one Ig molecule or different Ig isotypes because native electrophoresis itself is not good enough to prove monospecific protein molecules in one band on the gel. To ascertain the exact structural properties of fish IgG, two-dimensional electrophoresis is being conducted in our laboratory.

On the immunoblot analysis, two heavy chains were clearly observed, whereas a light chain was not done by rabbit anti-carp IgG, suggesting a possibility that fish light chains might not contain immunodominant epitopes to elicit a humoral immune reaction against rabbit. Moreover, unlike mammalian, fish light chains did not appear to be clearly stained with Coomassie blue on the gel, indicating that light chains might contain a large amount of carbohydrate or other molecules. It is, therefore, likely that the prothetic



groups such as carbohydrate or lipid around protein epitopes hinder an induction of immune reaction.

Although tilapia, *Oreochromis niloticus* has been developed quite differently from carp, *Cyprinus carpio*, the electrophoretic patterns of serum proteins from each fish were nearly identical. Furthermore, rabbit antibodies against carp Ig recognized tilapia Ig as well as Israeli carp Ig with a same degree. The result suggests that electrophoretic band patterns of fish serum proteins can be a good tool of classifying fish species. Although specific features of the immune response may vary widely between piscine species, the humoral immune response does share some basic features in form and function with that of mammals. These similarities include basic Ig structure, the cellular requisites for the induction of antibodies, and the role played by these antibodies in such activities as neutralization, complement fixation and opsonization. Many teleosts have been reported to possess only high molecular weight IgM which has a molecular weight of about 700 kd (Amemiya and Litman, 1990; Pilstrom and Petersson, 1991), and a molecular weight shift during the immune response has not been reported. However, some teleosts possess low molecular weight Igs which are found to be either a monomeric form of high molecular weight Ig or to be a physicochemical form distinct from high molecular weight Ig (Clem and McLean, 1975; Warr, 1983). In the present study, we investigated if a molecular weight shift during the immune response occurs on carp Ig structure. While, as time elapses following antigen challenge, the amount of some serum proteins were changed and other protein bands appeared, no molecular weight shift of carp Ig was observed on immunoblot analysis. The result suggests that carp Ig is not switched into other kinds of Ig isotypes.

In flow cytometry analysis, rabbit antibodies which reacted with tilapia serum Igs did not recognize tilapia cells, indicating that immunodominant epitopes present on serum Igs and cell surface Ig receptors are not identical. Expectedly, the rabbit antibodies recognized common carp mononuclear cells other than Israeli carp cells, suggesting that both fishes share same Ig structural properties irrespective of serum Igs or cell surface Ig receptors.

In conclusion, structural properties of carp serum Igs were characterized on the basis of an electrophoretic mobility and a flow cytometry analysis. It is crucial to obtain and characterize pure fish Igs to produce reagent antibodies as tools for the study of their specific immune responses.

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