# Production of Monoclonal Antibodies (Mabs) Against Surface Antigens on Israeli Carp Lymphocytes and Their Applications

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**ABSTRACT** : In fish both humoral and cell mediated immune responses have been reported whereas antibodies recognizing specific cellular populations have not yet been developed except for ones recognizing surface Ig molecules on B lymphocytes. Our aim was to develop and characterize monoclonal antibodies (Mabs) specific for the immune-related cells. Mabs were produced by fusion of myeloma cells (SP2/0) with Balb/c mouse spleen cells previously sensitized against Israeli carp (I. carp) kidney mononuclear cells. We obtained 44 Mabs positively reacting with I. carp kidney mononuclear cells and partially characterized 7 Mabs in the morphological and mitogen-based proliferative aspects. Fluorescence-activated cell sorter (FACS) analysis against I. carp kidney cells by using 7 different Mabs showed 80.3% for ICK 17-4, 65.1% for ICK 2-3, 64.1% for ICK 25-1, 67.5% for ICK 22-1, 70.8% for ICK 16-2, 76.8% for ICK 13-2, 79.7% for ICK 11-1. Panning method was used for the isolation of Mabs specific mononuclear carp spleen cells followed by Wright's stain. The stained cell populations were identified as monocytes (ICK 17-4, ICK 2-3, ICK 25-1, ICK 22-1 and ICK 16-2), lymphocytes (ICK 11-1), and a mixed cell population of monocytes and lymphocytes (ICK 13-2). In cell proliferation assay, monocytes purified by ICK 17-4, 2-3 and 22-1 efficiently responded to Con A and PHA, while ones separated by ICK 25-1 did not react with any mitogens. Lymphocytes isolated by ICK 11-1, though it is not known whether they are T or B cells, were more responsive to Con A than PHA or LPS, suggesting that fish immune cells are somewhat different from mammalian cells in responding to mammalian T or B cell mitogens. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 8 : 1179-1187)***

**Key Words** : Israeli Carp, Mabs, Cell Proliferation, Wright's Stain, Lymphocytes

## INTRODUCTION

Phylogenetically, fish represent the earliest animals to possess specific and non-specific immune responses. The distinction between vertebrates and invertebrates appears to represent the largest dichotomy in animal defenses (Klein, 1989; Secombes, 1991). Teleost fish possess both humoral and cell-mediated immunity (Graham and Secombes, 1990). Specific immune responses are characterized by the presence of lymphocytes that can proliferate in response to specific antigens, generating both effector and memory cell populations. The existence of various leucocyte subpopulations in teleost fish was reported on the basis of cell morphology (Ellis, 1977). Teleost fish have lymphoid cells which, based upon functioning properties, can be considered as B-lymphocytes, helper T-lymphocytes and accessory cells with reasonable confidence (Clem et al., 1991).

Israeli carp (*Cyprinus carpio* L.) kidney contains morphologically/functionally distinct subpopulations of mononuclear cells seemingly analogous to their mammalian counterparts, e.g., T-lymphocytes, B-lymphocytes, monocytes or neutrophils (Secombes et al., 1983; Clem et al., 1985). Although these data argue strongly for the existence of T- and B-lymphocytes in I. carp, it has not been clearly identified whether T- and B-lymphocytes have their own specific cell surface markers.

In the last decade, studies on the teleost immune system have been greatly benefited by the production of monoclonal antibodies (Mabs) which have been used as tools for studying specific markers present on fish leucocyte cell surface (Secombes et al., 1983; Sanchez et al., 1993; Nakayasu et al., 1998; Miller et al., 1987). The usefulness of Mabs stems from three characteristics: its specificity, homogeneity, and ability to be produced in unlimited quantities. Mabs against other subpopulations of leucocytes have been reported for channel catfish T-lymphocytes (Ainsworth et al., 1990), channel catfish non-specific cytotoxic cells (Evans et al., 1988), carp thymocytes (Scapigliati et al., 1995), rainbow trout thrombocytes and granulocytes (Slierendrencht, 1995) and carp thrombocytes (Nakayasu et al., 1997). Nonetheless, however, the presence of specific fish immune cell subpopulations have not yet been identified by Mabs.

The present study was performed in an attempt to develop specific Mabs against I. carp kidney mononuclear cells and characterize antibody-purified fish immune cells in phenotypical and functional aspects. The strategy for this

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study involved three major phases. The first is identification of Mabs against antigens present on I. carp mononuclear cells. The second is the use of Mabs to separate immune cell subpopulations. The third is phenotypical and functional characterization of the antibody-purified fish immune cells.

A better understanding of functional fish immune cells will be helpful to evaluate and develop effective vaccines for fishes and to monitor the course of therapy in infected fishes.

## MATERIALS AND METHODS

### Fish and cells

Israeli carp (I. carp), approximately 1 kg (±50 g), common carp, tilapia, catfish and rainbow trout were obtained from the aquaculture facility in the department of Marine Biomedical Science, Kunsan National University, Kunsan, Korea. I. carp were anaesthetized with MS222 (1/10,000). The I. carp kidney was aseptically dissected through a ventral incision, and then transferred to DMEM (Gibco BRL, USA) media supplemented with antibiotic/antimycotic (Gibco BRL), 3.7 g sodium bicarbonate (Sigma, USA), and 5% bovine calf serum (BCS, Gibco BRL, 5% BCS-DMEM). I. carp kidney cell suspension was prepared by rubbing kidney with two slide glasses. The cells were washed twice by centrifugation at 1200 rpm for 8 min, and the cell pellet was resuspended in 14 ml of DMEM. The cell suspension was overlayed on 7 ml of Histopaque-1077 (Sigma) and then centrifuged at 2500 rpm for 40 min to separate I. carp kidney mononuclear cells. The I. carp mononuclear cells were collected at the interface between the DMEM and Histopaque-1077, and then washed twice with DMEM. The mononuclear cell pellet was resuspended in 5% BCS-DMEM, and then cell viability (>95%) was checked by tryphan blue exclusion method.

### Production of Mabs

I. carp mononuclear cells were washed twice with phosphate buffered saline (PBS) and resuspended in PBS. Balb/c mouse was immunized with $10^6$ of I. carp kidney mononuclear cells in 200 μl of PBS by intraperitoneal (I. P.) injection, and boosting was given 14 and 28 days later. On the third day after final injection, the spleen was isolated from the mouse. The mouse spleen cell suspension was prepared by rubbing spleen with two slide glasses with serum-free DMEM.

Myeloma cells (SP2/0), cultured in 10% fetal bovine serum (FBS, Gibco BRL)-DMEM (10% FBS-DMEM), were harvested and washed by centrifugation with serum-free DMEM. Viability of mouse spleen cells and myeloma cells was checked by tryphan blue exclusion method.

Hybridomas were produced by fusion method using polyethylene glycol (Köler and Milstein, 1975). Briefly, $1\times10^8$ of spleen cells and $1\times10^7$ of myeloma cells were mixed in a 50 ml conical tube (Corning, USA) and the medium was removed. One milliliter of 50% polyethylene glycol (PEG, Sigma) was added to the mixed cells by dropping within 1 min at 45°C. The fused cells were washed twice at 25°C with 20 ml of serum free-DMEM followed by addition of 21 ml of HAT (Sigma)-media (15% FBS-DMEM supplemented with 2% (V/V) HAT supplement). The fused cells were cultured in three 96-well plates (Corning) with mouse macrophage from ascitic fluid. Hybridoma supernatants were screened by FACScan analysis to identify the production of antibodies against I. carp kidney mononuclear cells. Hybridoma clones secreting antibodies were established by limiting-dilution method. The established hybridomas were stored in a liquid nitrogen tank and the supernatants were used for all experiments performed in this study.

Isotyping for Mabs was performed according to the manufacturer's recommendation by using a commercial kit (ISO-1, Sigma). Briefly, concentrated supernatants containing Mabs to be tested for isotyping were added to the holes in 1% agarose gel followed by incubation of goat anti-mouse IgM, IgA, IgG1, IgG2a, IgG2b and IgG3, respectively. Isotypes of Mabs were determined by the precipitated line formed between Mab and one of six different kinds of goat anti-mouse Igs.

### Purification of I. carp immunoglobulins (Igs)

The fish were injected intraperitoneally with 0.4 mg of mouse IgG (Sigma) emulsified with Freund's complete adjuvant (Sigma) and boosting was given four weeks later. Three to five days later, blood was collected from the caudal vein of I. carp and allowed to clot for overnight at 4°C. Serum was obtained by centrifugation at 2,500 rpm for 8 min.

One milliliter of mouse IgG-sepharose 4B beads (Sigma) were added to a 5 ml syringe and allowed gradually to pack by gravity. The beads were washed with 30 ml of PBS and 10 ml of elution buffer (0.1 M glycine-buffer, pH 11), and then the beads were equilibrated with 50 ml of PBS. One milliliter of immunized I. carp serum was applied to the mouse IgG-sepharose 4B column. After 20 min, column was washed with 500 ml of PBS. The I. carp anti-mouse IgG (I. c $\alpha$ MIgG) were eluted from the mouse IgG-sepharose 4B column by elution buffer, and then concentrated to 1 ml. The I. c $\alpha$ MIgG fraction was dialyzed with PBS for 24 h at 4℃. The purity was checked by SDS-PAGE.

### Preparation of rabbit anti-I. carp Igs (R $\alpha$ I. cIgs)

Two female rabbits were injected with purified I.

c $\alpha$ MIgG emulsified with Freund's complete adjuvant (FCA, Sigma) at 0.5 ml per rabbit. Two weeks later, the rabbit was boosted with 0.1 mg of I. c $\alpha$ MIgG emulsified with Freund's incomplete adjuvant (FIA, Sigma) at 0.5 ml per rabbit, and then the final boosting was performed with 0.1 mg of I. c $\alpha$ MIgG in 0.5 ml of PBS per rabbit. Three days after final boosting, the rabbits were sacrificed and serum was separated by centrifugation. The purified serum was used for staining of cells separated by Mabs.

**Fluorescence-activated cell scan (FACScan) analysis**

Kidney and spleen mononuclear cells were obtained from I. carp, common carp, tilapia, catfish and rainbow trout, respectively. The mononuclear cells were washed twice with DMEM. The mononuclear cells were adjusted to $2\times10^6$ of cells in 100 μl of DMEM and then incubated with 100 μl of hybridoma supernatants for 30 min at 4°C. After washing cells bound with primary Mabs, 100 μl of fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (G $\alpha$ MIgG-FITC, Promega, USA) were added followed by incubation for 30 min at 4℃. The cells were fixed by 200 μl of PBS containing 2% (W/V) paraformaldehyde. The fluorescence-positive cell populations were subsequently monitored on a flow cytometer (FACScan, Becton-Dickinson, USA). From each, $5\times10^3$ of cells per sample were collected for FACS-analysis. Histograms for FL1 (FITC) were used to analyze the fluorescence-positive cell populations.

**Purification of fish immune cells by Mabs**

The I. carp kidney mononuclear cells were adjusted to $2\times10^7$ of cells in 100 μl of serum free DMEM and the cell suspensions were added into the wells of 96-well round bottom plates (Corning), and then hybridoma supernatants were added to the wells containing I. carp kidney mononuclear cells. The mixtures of cells and supernatants were incubated for 1 h at 4℃. The Mabs-reacted cells were washed twice with DMEM. HAT-media were used as a negative control.

Polystyrene petri-dishes (Corning) were coated with 5 ml of DMEM containing 50 μg of goat anti-mouse IgG (G $\alpha$ MIgG, Sigma) for overnight at 4℃. The G $\alpha$ MIgG-coated petri-dishes were washed twice with Hanks' balanced salt solution (HBSS, Sigma) and then non-specific binding sites were blocked by HBSS containing 2% (w/v) bovine serum albumin (BSA, Sigma) for 1 h at 37℃. The Mab-reacted cells in 5 ml of DMEM were seeded onto G $\alpha$ MIgG-coated petri-dishes. The cells were incubated for 1 h at 4°C after which non-adherent cells were washed off the petri-dish, and then panned cells were harvested by pipetting with 1 ml micro pipette. All steps were performed aseptically. The collected cells were used in Wright's staining and mitogen-induced proliferation assay. Furthermore, the panned cells were incubated with R $\alpha$ I. cIgs and goat anti-rabbit IgG-FITC (G $\alpha$ RIgG-FITC, Promega) to investigate how many surface Igs positive cells were present in the panned cells.

**Mitogen-induced cell proliferation assay**

The panned cells were adjusted to $3\times10^6$ of cells/ml in 10% FBS-DMEM, and then $3\times10^5$ of cells/100 μl were seeded into wells of a 96 well plate. The 100 μl of 10% FBS-DMEM containing 20 μl of phytohemagglutinin (PHA, Sigma)/ml, 10 μl of Concanavalin A (Con A, Sigma)/ml, or 200 μg of lipopolysaccharide (LPS, Sigma)/ml were added to the wells containing panned cells which were purified by various kinds of Mabs. The cells were incubated under saturated humidity atmosphere of 7% $CO_2$ at 37°C. Ten percent FBS-DMEM was used as a negative control.

The cells were incubated for a further 72 h prior to the addition of 0.5 μCi [$^3$H] thymidine into the wells containing proliferating cells. After 12 h, the cells were harvested using an automash 2000 cell harvester (Dynatech, USA) trapping their DNA onto glass filtermats. Dried filter circles were placed in plastic scintillation counter vials (Packard, USA). All cpm values were subtracted by the background cpm values of non-stimulated cultures. All samples were prepared in triplicate.

## RESULTS

**Production of MAbs**

After spleen-myeloma fusions, 547 hybridomas (>95% of the total wells) were obtained, and then they were screened by FACScan. Forty four different clones (8% of the total hybridoma clones) appeared to positively react with I. carp kidney mononuclear cells. Seven Mabs were selected for further characterization and named ICK 17-4, ICK 2-3, ICK 25-1, ICK 22-1, ICK 16-2, ICK 13-2, and ICK 11-1.

The isotype classes of the seven selected Mabs are shown in table 1.

**FACScan analysis**

The 7 selected Mabs responded efficiently with I. carp kidney mononuclear cells. The Mabs were, therefore, used

**Table 1.** Isotypes of monoclonal antibodies

| Clones (ICK) | Isotypes | Clones (ICK) | Isotypes |
|---|---|---|---|
| 17-4 | Ig G1 | 16-2 | Ig M |
| 2-3 | Ig G2b | 13-2 | Ig M |
| 25-1 | Ig G1 | 11-1 | Ig M |
| 22-1 | Ig G1 | | |

in FACScan analysis. Figure 1 shows histograms of fluorescence-positive I. carp kidney mononuclear cells. FACS analysis against I. carp kidney mononuclear cells by using 7 different Mabs showed 80.3% for ICK 17-4, 65.1% for ICK 2-3, 64.1% for ICK 25-1, 67.5% for ICK 22-1, 70.8% for ICK 16-2, 76.8% for ICK 13-2, and 79.7% for ICK 11-1, respectively.

In order to investigate whether 7 different Mabs cross-react with other kinds of fish immune cells, FACS analysis was performed with kidney and spleen cells from I. carp, common carp, catfish, tilapia, and rainbow trout. All antibodies positively responded with both I. carp and common carp with different percentages (table 2). Interestingly, ICK 16-2 reacted with spleen cells from catfish (table 2). On the other hand, mouse polyclonal antibodies against I. carp kidney cells strongly cross-reacted with kidney and spleen cells from catfish, spleen cells from rainbow trout, and kidney cells from tilapia.

**Purification of fish immune cells by Mabs**

Mabs-reacted I. carp kidney mononuclear cells were positively separated by panning method followed by Wright's stain. The stained cells were observed by optical microscopes. The Wright's stained cell populations were

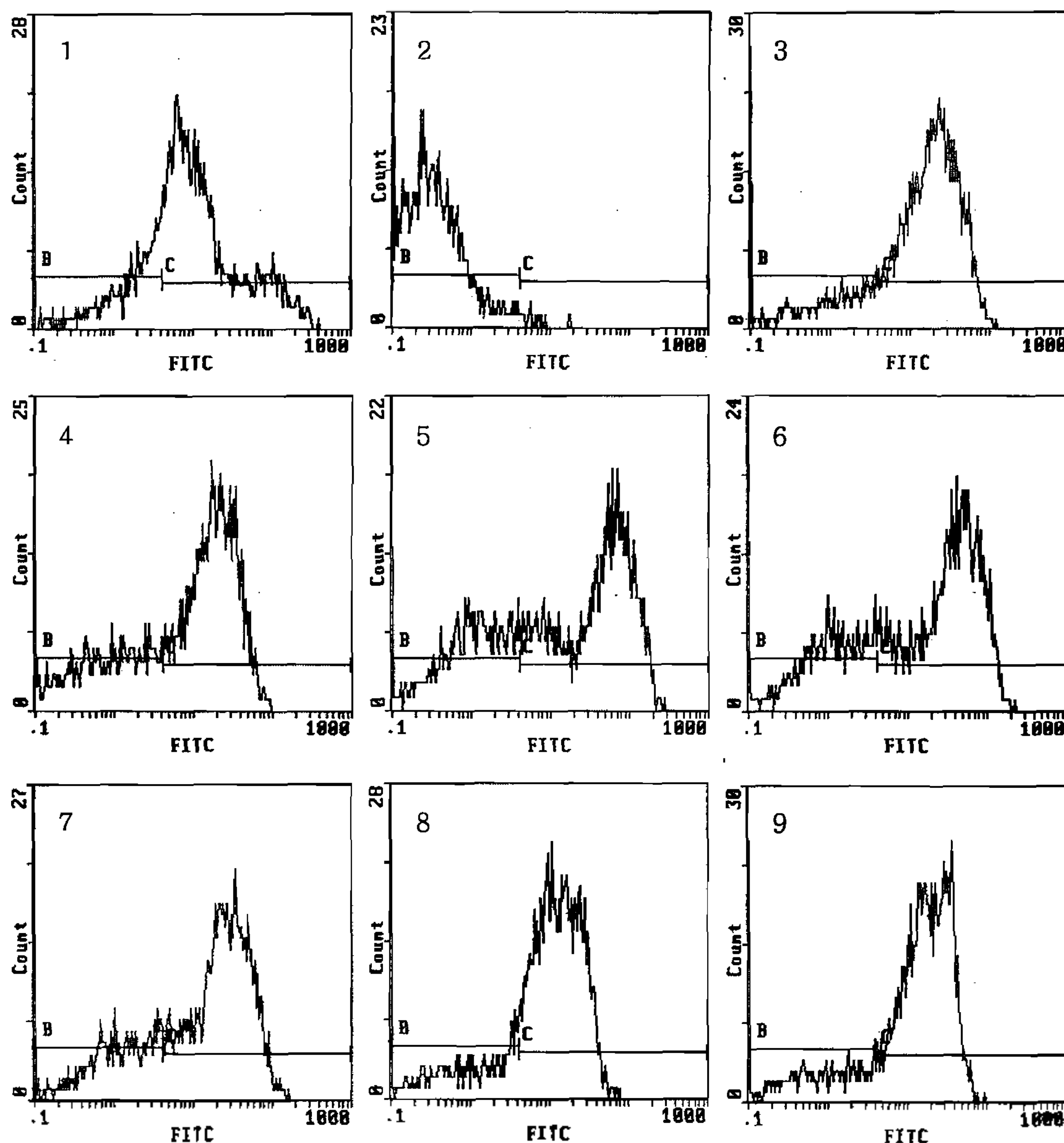


**Figure 1.** FACScan analysis for I. carp kidney mononuclear cells. Panel 1, positive control (mouse polyclonal antibodies against I. carp kidney mononuclear cells); Panel 2, negative control (HAT media-reacted cell population); Panels 3-9, seven different Mabs (ICK 17-4, ICK 2-3, ICK 25-1, ICK 22-1, ICK 16-2, ICK 13-2 and ICK 11-1, respectively)-reacted cell populations. The histograms show the population of fluorescence (FL1) positive I. carp mononuclear cells.

identified as monocytes (ICK 17-4, ICK 2-3, ICK 25-1, ICK 22-1 and ICK 16-2), lymphocyte (ICK 11-1), and a mixed cell population of monocyte and lymphocyte (ICK 13-2), respectively (table 3). Figure 2 shows representative Wright's staining of cells (1, normal kidney mononuclear cells; 2, monocytes; 3, a mixed cell population of lymphocytes and monocytes; 4, lymphocytes). To study how many panned cells were surface Ig positive, FACScan analysis was performed with R I. cIgs plus G RIgG-FITC. Unexpectedly, all panned cell populations were surface Ig positive except for one separated by ICK 13-2 (figure 3).

**Mitogen-induced cell proliferation assay**

Thymidine incorporation assay was performed to identify how each purified cell population responded to three different kinds of mitogens, PHA, Con A and LPS (figure 4). Monocytes separated by ICK 17-4, 2-3 and 22-1 were efficiently reactive to Con A and PHA. Monocytes separated by ICK 25-1 failed to react with any mitogens used. The mixed cell population of monocytes and lymphocytes (ICK 13-2) showed a similar pattern of cell proliferative activity to that of monocytes isolated by ICK 17-4. Lymphocyte cell population (ICK 11-1) was more reactive to Con A than PHA or LPS.

The results suggest that fish immune cells are quite different from mammalian cells in proliferating to mammalian T- or B-lymphocyte mitogens.

## DISCUSSION

Studies concerning the biology and development of the fish immune system have been boosted by monoclonal antibody technology, which has led to the generation of hybridomas secreting antibodies targeted toward leucocyte populations including B-lymphocytes from carp (Secombes et al., 1983; Koumans-van Diepen et al., 1994), trout (Thuvander et al., 1990), sea bass (Navarro et al., 1993), catfish neutrophils (Bly et al., 1990), and catfish non-specific cytotoxic cells (Evans et al., 1988).

In fish the kidney is a hematogenous (MacArthur et al., 1983) and lymphoid (Tatner and Findly, 1991) organ responsible for the production and residence of immune cells. This study was performed to identify subpopulations of fish immune cells with different surface markers. Thus,

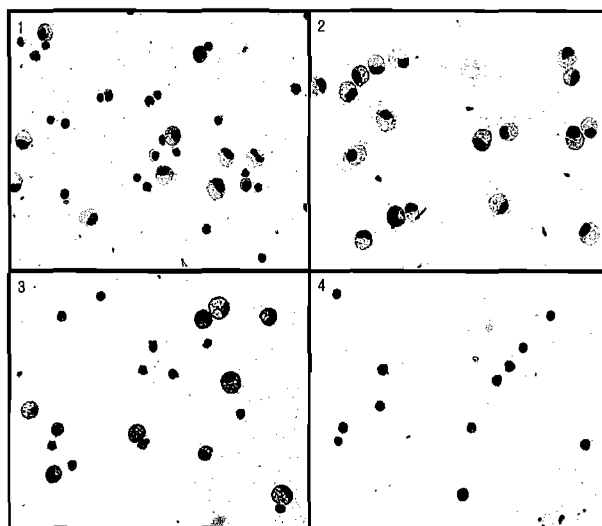


**Figure 2.** Representative Wright's staining patterns of cells isolated by panning method. Panel 1, normal kidney mononuclear cells; Panel 2, monocytes isolated by ICK 25-1; Panel 3, the mixed cell population of monocytes and lymphocytes isolated by ICK 13-2; Panel 4, lymphocytes isolated by ICK 11-1.

**Table 2.** Cross-reactivity of Mabs against five different fish immune cells

| | | Polyclonal Ab | | Monoclonal Ab | | | | | | (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Fish | Organ | P[1] | N[2] | 17-4 | 2-3 | 25-1 | 22-1 | 16-2 | 13-2 | 11-1 |
| I. carp | Kidney | 40.7[3] | 0.0 | 48.9 | 30.8 | 29.1 | 34.9 | 31.5 | 36.5 | 35.9 |
| | Spleen | 55.1 | 0.2 | 64.5 | 45.5 | ND[4] | 41.2 | 29.7 | 54.3 | 56.3 |
| Carp | Kidney | 77.8 | 1.6 | 85.2 | 70.6 | 63.5 | 80.7 | 73.9 | 77.6 | 84.2 |
| | Spleen | 94.1 | 1.0 | 89.3 | 79.0 | 66.4 | 0.4 | 77.3 | 90.1 | 90.0 |
| Catfish | Kidney | 72.3 | 0.7 | 0.5 | 0.40 | 1.0 | 0.4 | 0.9 | 0.8 | 0.9 |
| | Spleen | 76.0 | 0.8 | 2.3 | ND | 4.3 | 1.4 | 63.8 | 4.6 | 2.7 |
| Tilapia | Kidney | 16.0 | 0.0 | 0.0 | ND | 0.6 | 0.2 | 0.2 | 0.2 | 0.4 |
| | Spleen | 6.0 | 0.1 | 0.2 | 0.1 | 0.0 | 0.4 | 0.6 | 0.2 | 0.0 |
| R.trout | Kidney | 6.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| | Spleen | 61.1 | 0.3 | 1.1 | 0.5 | 0.8 | 1.0 | 0.7 | 0.2 | 6.5 |

[1] Positive control; mouse antibodies against I. carp kidney mononuclear cells.

[2] Negative control; HAT media.

[3] Percentages of cell population labelled with specific antibodies (FL1).

[4] Not determined.

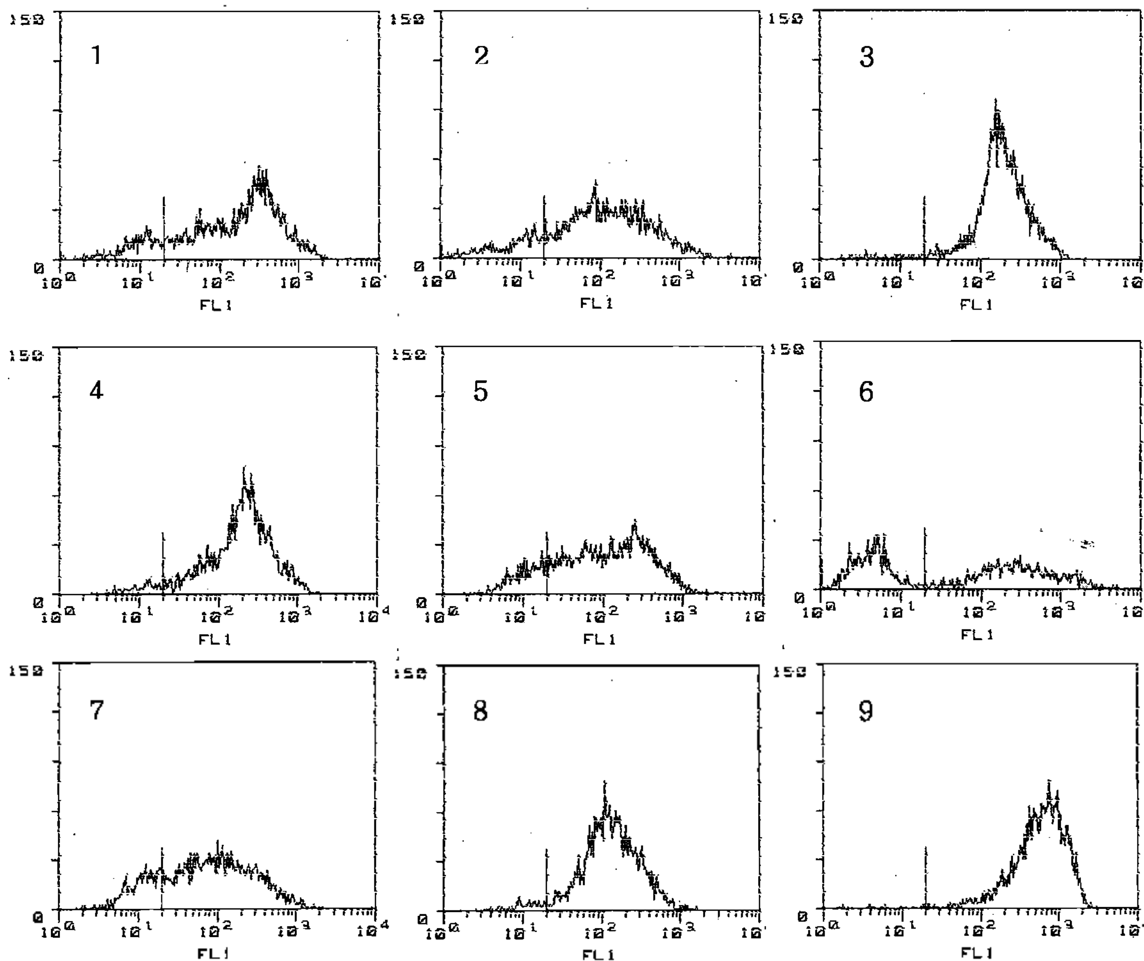


**Figure 3.** FACScan analysis for rabbit anti-I. carp Igs positive cell populations among panned cells. Panels 1-7, cells purified by ICK 17-4, ICk 2-3, ICK 25-1, ICK 22-1, ICK 16-2, ICK 13-2 and ICK 11-1, respectively; Panel 8, I. carp kidney mononuclear cells; Panel 9, I. carp spleen mononuclear cells.

Mabs against I. carp kidney mononuclear cells were developed. FACScan analysis shows that the produced Mabs recognize I. carp kidney mononuclear cells at 80.3% for ICK 17-4, 65.1% for ICK 2-3, 64.1% for ICK 25-1, 67.5% for ICK 22-1, 70.8% for ICK 16-2, 76.8% for ICK 13-2, 79.7% for ICK 11-1, 70.8% for positive control and 1.2% for negative control. It was observed that Mabs produced in this study recognized various subpopulations in I. carp kidney mononuclear cell. The result suggests that specific cell subpopulations might be purified by Mabs for further characterization of fish immune system.

Polyclonal and monoclonal antibodies against I. carp kidney mononuclear cells strongly cross-reacted with normal carp kidney mononuclear cells with some different ratios, suggesting that a genetic moiety of I. carp has been originated from normal carp. Other fish cells failed to show a positive reaction with other Mabs except for ICK 16-2 which could not recognize kidney cells but did recognize spleen cells from catfish. We can, therefore, speculate that ICK 16-2 specific mononuclear cells are more residing in spleen than kidney of catfish. On the other hand, positive control antibodies appeared to cross-react with both kidney and spleen cells from catfish and only spleen cells from rainbow trout.

Indirect panning technique used in the present study proved to be highly effective in separating Mab specific I.

**Table 3.** Morphological patterns of I. carp kidney mononuclear cells isolated by Mabs

| Mabs (ICK) | Purified cells |
|---|---|
| 17-4 | Monocyte |
| 2-3 | Monocyte |
| 25-1 | Monocyte |
| 22-1 | Monocyte |
| 16-2 | Monocyte |
| 13-2 | Lymph/Mono[1] |
| 11-1 | Lymphocyte |

[1] The mixed cell population of monocytes and lymphocytes

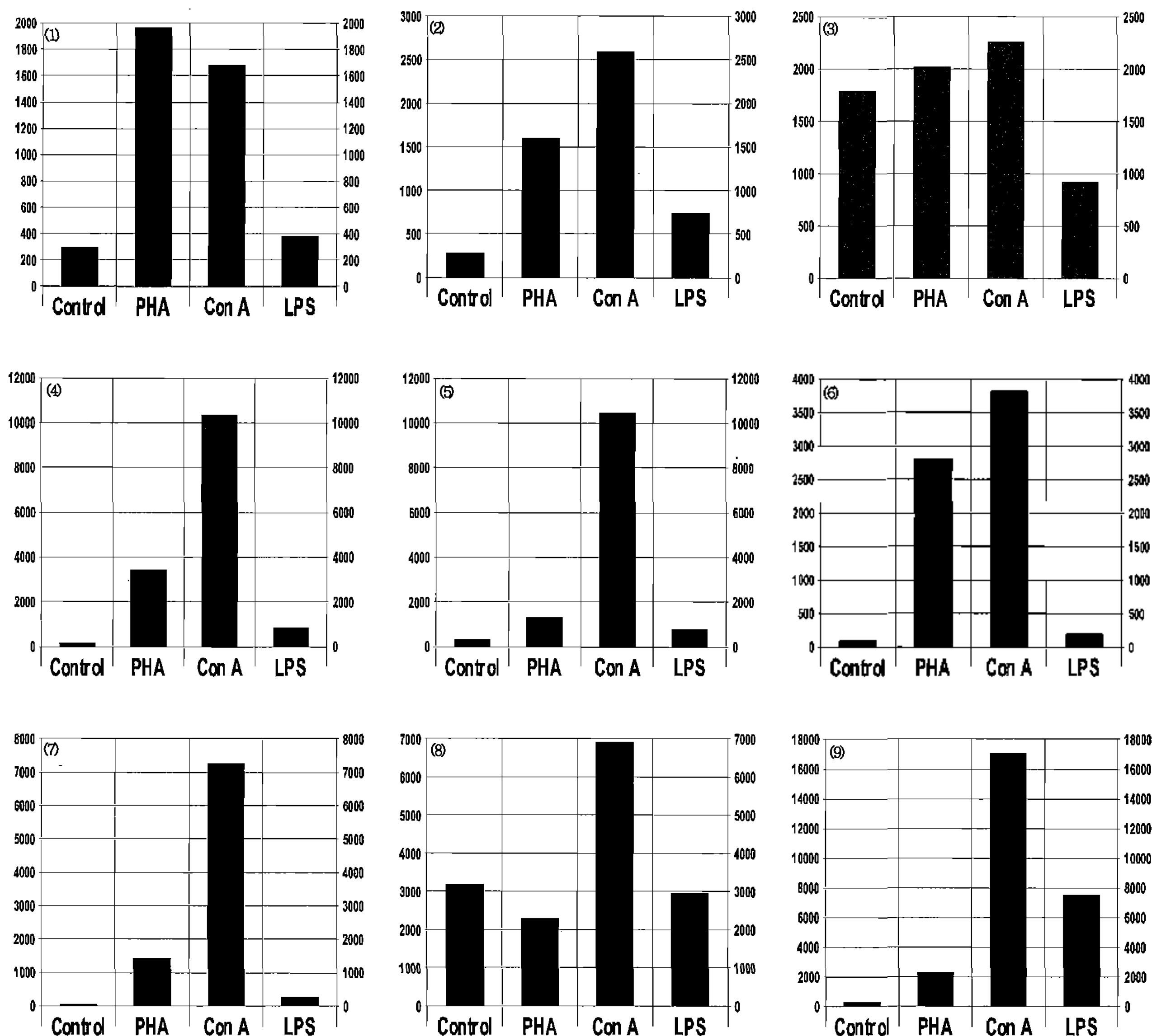


**Figure 4.** Mitogen-induced thymidine incorporation assay for each cell population isolated by Mabs. Panels 1-5, monocytes purified by ICK 17-4, ICK 2-3, ICK 25-1, ICK 22-1, and ICK 16-2, respectively; Panel 6, the mixed cell population of monocytes and lymphocytes purified by ICK 13-2; Panel 7, lymphocytes purified by ICK 11-1; Panel 8, I. carp kidney mononuclear cells; Panel 9, mouse spleen mononuclear cells. Cultures were pulsed with 0.5 μCi [$^3$H] thymidine for 72 h before cell harvest. Radioactivity was measured by liquid scintillation spectrometry and the results are expressed as cpm incorporation of triplicates.

carp kidney mononuclear cells. The panning technique had been shown to be highly effective in separating rainbow trout sIg$^+$ and sIg$^-$ PBL (Graham and Secombes, 1990). In fact, some contamination of other cell populations could not be excluded. To identify morphological properties of panned cells and their purity, Wright's stain was performed. As a result, it was ascertained that no other cells were contaminants in the course of separating Mab-specific mononuclear cells. For further convincing results, however, highly improved cell separating methods such as magnetic cell separation system (MACS) or fluorescence activated cell sorter (FACS) should be used in future.

Flow cytometric analysis of FS/SS profiles of leukocytes in the carp kidney resulted in three fractions: lymphocytes, monocytes and granulocytes (Verburg-van Kemenade et al., 1994). As a result of Wright's staining, panned I. carp mononuclear cells were distinguished into three groups: monocyte, lymphocyte, and a mixed population of monocyte and lymphocyte. Identification of each cell population was based on morphological criteria (Cenini, 1984, and Imagawa, 1989). The ICK 17-4, ICK 2-3, ICK 25-1, ICK 22-1 and ICK 16-2 are specific to

monocytes. ICK 13-2 is specific to a mixed population of monocytes and lymphocytes, and ICK 11-1 to lymphocytes. Although the cells isolated by ICK 11-1 were morphologically lymphocytes, more studies should be done to identify whether they are T- or B-lymphocytes. The cell population purified by ICK 13-2 was observed as a mixture of monocytes and lymphocytes, indicating that ICK 13-2 recognized the same cell surface markers commonly present on the both cell populations. Monocytes separated by each different Mab clone cannot be necessarily expressing identical surface molecules. In other words, some other monocyte-specific surface molecules might be recognized by other different Mabs. In order to know exact surface molecules recognized by Mabs, an experiment using cell surface molecule labelling techniques has been made in our laboratory. Kidney is hematopoietic organ in teleost fishes, and antigen specific antibodies are produced from the kidney, suggesting that mammalian-like B lymphocytes are mostly present in the organ. Thus, we tried to identify how many percentages of B-like lymphocytes producing Igs are populated in whole kidney cells and Mab-panned cells. Surprisingly, kidney mononuclear cells are all surface Ig positive (95.9%). Furthermore, Ig positive percentages of each Mab-purified cell populations from kidney ranged from 54.5% to 97.3%. The result suggests two possibilities. One is that I. carp kidney mononuclear cells are all cell surface Ig positive irrespective of Ig-producing ability, in other words, properties of mammalian B-lymphocytes secreting Igs may be not the same as ones of I. carp B-like lymphocytes. The other possibility is an experimental failure. However, the first possibility was exclusive because the result was consistently reproducible (n=5). In the case of ICK 16-2, only 54.5% of cells are surface Ig strong positive with clean two peaks in histogram of FACS analysis. It is, therefore, worthwhile to separate surface Ig positive cell population from ICK 16-2-purified cells and characterize their function, i. e., ability of producing Igs.

Thymidine incorporation assay was performed to investigate whether panned lymphocytes or monocytes nonspecifically respond to three kinds of mitogens, e.g., LPS, PHA and Con A. Most panned cell populations appeared to respond to Con A. Some purified cells (cell populations separated by ICK 17-4, ICK 2-3, ICK 22-1 and ICK 13-2) showed moderate responses with PHA. On the other hand, LPS failed to proliferate all cell populations. The results indicate that the properties of fish mononuclear cells responding to mammalian mitogens are quite different from those of mammalian cells. In fact, even panned I. carp monocytes were efficiently proliferated by Con A or PHA. However, the possibility of contamination with other cells could not be excluded because cells stained by Wright's stain were layered on slide glass in a restricted area. Thus, highly improved cell separating systems,. e. g., Mini MACS or FACS are required to obtain convincing results.

In summary, several Mabs against I. carp mononuclear cells were produced and seven selected Mabs were used in separating Mab-specific mononuclear cells. The purified cell populations appeared as monocytes, lymphocytes or mixed cell population of monocytes and lymphocytes and they showed quite different proliferative patterns from mammalian cells. Further studies should be performed to identify the exact immune mechanisms occurring in teleost fishes. A better understanding of the fish immune system using the purified various kinds of immune-related cells will be helpful to design effective fish vaccines and monitor fish diseases in the course of therapy.

## ACKNOWLEDGEMENT

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