Korean J Biol Sci 4: 341-345, 2000

Characterization of B-, T-, and NK-like Cells in Nile Tilapia *(Oreochromis nilotica)*

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Key Words:
SIg^+ cells
Head kidney
T-cell proliferation
NK cell

It has been very difficult to develop and evaluate efficient fish vaccines because fish immune cells have not been properly characterized. In this study, we investigated the cell-mediated immunological properties of B- and T-like cells in Nile tilapia (*Oreochromis nilotica*). Surface immunoglobulin negative (sIg^-) cell population proliferated in response to mammalian T-cell mitogens PHA and Con A, while surface immunoglobulin positive (sIg^+) cells responded to the B-cell mitogen LPS. The sIg^- cells from hemocyanin (HC)-immunized Tilapia, compared to the non-immunized control, reacted more to PHA than to Con A. Unexpectedly, antigen (Ag)-specific response was observed in both sIg^- and sIg^+ cells. Regardless of HC immunization, whole leukocytes from a head kidney of fish showed natural killer (NK) cell activity. Especially, NK cell activity was much higher in sIg^- cells than in sIg^+ cells, indicating the possibility that fish NK cells were not at least associated with sIg^+ cell population and not activated by Ag. Further understanding of functional fish immune cells will help to evaluate and develop effective vaccines for fishes and to monitor the course of therapy in infected fishes.

In Mammals, T and B lymphocytes are characterized by specific markers, such as T-cell receptor (TCR) and immunoglobulin (Ig) on their surface, respectively. Clem et al. (1991) reported that teleost fish possesses lymphocyte populations analogous in many respects to the mammalian T- and B-cells. Various functional studies were performed *in vitro* on heterogeneous fish lymphocytes obtained from different organ sources (Etlinger et al., 1976) and a hapten-carrier effect was demonstrated *in vivo*, indicating that production of antibody by B cells against a hapten required carrier-specific (putative T- cell) cooperation (Avtalion et al., 1976; Stolen and Makela, 1976; Ruben et al., 1977). This view of a T-cell/B-cell dichotomy in fish has recently been strengthened by cell separation techniques utilizing monoclonal antibodies (Sizemore et al., 1984; Graham and Secombes, 1990a). In fishes, monoclonal antibodies raised against homologous serum immunoglobulin have been used to label and separate surface immunoglobulin positive (sIg^+) B lymphocytes from the surface immunoglobulin negative (sIg^-) population (which comprises putative T cells together with other leukocyte types).

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Panning method was frequently used to separate fish Ig^+ cells from Ig^- cells for *in vitro* studies (Sizemore et al., 1984). When using anti-fish Ig, the antibody binds to sIg^+ cells and leaves the nonadherent sIg^- cells free in suspension. The third cell type important for the immune response, the monocyte/macrophages, can be separated on the basis of its adherent property. As we previously reported (Choi, 1997), purified fish sIg^+ cells were B-like cells which produced specific antibodies against bovine serum albumin (BSA).

However, in order to induce an antibody response to a protein antigen, the antigen must be processed by accessory cells (macrophages) and presented on the cell surface to the T cell (Vallejo et al., 1991 and 1992), which in turn, elaborates requisite interleukins. These interleukins then provide the requisite signals and growth factors for B-cell differentiation (Caspi and Avtalion, 1984; Groundel and Harmsen, 1994). In addition to the population of $CD4^+$ helper cells which are effective in eliciting B-cell responses, other $CD4^+$ subpopulation mediate functions associated with specific cytotoxicity and local inflammatory (cytokine-induced) reactions. In this study, we investigated whether T- and B-like cells are also present in fish and function like mammalian cells. T-like cell population was negatively purified using rabbit antibody to fish Ig molecule because no specific T cell surface molecule has yet been identified.

Materials and Methods

Immunization of tilapia with hemocyanin

Twenty Nile tilapia (*Oreochromis nilotica*) (mean weight 220-400 g) were intraperitoneally immunized on day 0 with 300 μg of hemocyanin (HC) (Sigma) per 100 g body weight, in 0.2 ml of a 1:1 emulsion in Freund's complete adjuvant (FCA). Boosters were injected on days 14 and 30 with the same doses of HC in 0.2 ml of a 1:1 emulsion in Freund's incomplete adjuvant (FIA). Control fish were injected with FCA and FIA without the antigen on the same immunizing schedule. 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℃ for 2 h, and then at 4℃ overnight. The serum was separated, pooled, and stored in aliquots at -20℃ until used.

Purification of tilapia antibodies

Fish anti-HC antibodies were purified by affinity chromatography as described by Smith et al (1997). Briefly, 2 ml of HC-coupled Sepharose 4B beads were packed on a 7×1.0 cm syringe column, washed with 200 ml of 0.01 M phosphate buffered saline (PBS, pH 7.2) containing 0.5 M sodium chloride (equilibration buffer) and then eluted with 20 ml of 0.1 M glycine-NaOH (pH 11). After the column was re-equilibrated, 2 ml of the pooled immune fish serum were diluted 1:1 with equilibration buffer, 0.22 μm-filtered and applied to the column. Then 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 dialysed against PBS.

Production of polyclonal antibodies against fish Igs

A New Zealand white rabbit (male, 1.0-1.5 kg) was subcutaneously injected on day 0 with 100 μg of the affinity-purified fish Igs emulsified 1:1 in FCA, in a total volume of 1 ml per rabbit. Boosters were performed in the same way on days 14 and 30 with the antigens prepared in FIA, and on day 45 without the adjuvant. Specificity of the rabbit antisera was ascertained by western blotting. To purify fish sIg^+ lymphocytes, IgG fractions from rabbit antiserum were purified using a protein A column.

Purification of sIg+ cells from tilapia

Head kidney from tilapia was removed and prepared into single cell suspension. Fish monocytes were harested from the interface of Ficoll-Hypaque (Pharmacia) density gradient. The cells (1×10^8) isolated from Ficoll-Hypaque were incubated with 100 μl of rabbit α fIgM (1:200) for 30 min at 4℃. After the incubation, the cells were washed with 1% BSA-PBS twice followed by re-incubation with goat α rabbit IgG-magnetic beads for 15 min. Magnetically labelled cell suspension was applied on top of a Mini-Macs column (Miltenyi Biotec Inc.) allowed to pass through, and washed with 500 μl PBS. Effluent was harvested as sIg^- fraction. The column was removed from separation unit, placed on a tube, added with 1 ml of 1% BSA-PBS, and then magnetically labelled cells were flushed out with gentle pressure using a plunger. By FACScan (Becton Dickinson), the finally purified cells were identified as above 95% of sIg^+

Cell proliferation test

For antigen specific proliferation, aliquots of 100 μl DMEM (Gibco) medium containing 3×10^5 of primed sIg^+ or sIg^- cell populations were added to 96-well tissue culture plates (Costar) in the presence or absence of HC or chicken albumin (CA). Additionally, 1×10^5 of γ-irradiated (2000 rad) fish kidney cells were added followed by 72 h incubation. To study the effect of mitogens on the proliferation of purified fish lymphocytes, 100 μl aliquots of medium containing 5 μg /ml of phytohemagglutinin (PHA) (Sigma), Conconavalin A (Con A) (Sigma) or 50 μg/ml of lipopolysaccharide (LPS, Serotype 0127:B8) (Sigma) were added to sIg^+ or sIg^- cells (1×10^6 cells/well). The cells were then cultured for 72 h in 5% CO_2 at 25℃. Cultures were pulsed with 0.5 μCi [^{3}H]-thymidine (2 mCi; New England Nuclear Research Products) 8 h before harvesting onto glass fiber filters (Whatman). Results are expressed as mean cpm incorporation of triplicates.

Cytotoxicity by natural killer (NK) cell

Human erythroleukemia cell line K562 (donated by the Cell line Bank at the Cancer Reaearch Center, Seoul National University) was used as target cells in assays of NK cell cytotoxicity. Various numbers of the effector cells (100 μl), which were isolated from HC-primed or control tilapia kidney cells by a Ficoll- Hypaque density gradient, were added to a 100 μl target cell suspension containing 2×10^3 target cells in each well of 96-well round-bottom microtiter plates (Costar) and to different E/T ratios in triplicate. Spontaneous release wells contained chromium-labeled target cells in culture media only; maximum release wells contained target cells in 5% SDS. Following incubation for 5 h at 37℃ in a 5% CO_2 incubator, 100 μl supernatant was harvested and radioactivity was determined in a γ-counter (LKB 1272).

Results

Cell proliferative activity by mitogens

Thymidine incorporation assay was performed to identify whether fish B- or T-like cells nonspecifically respond to three kinds of mitogens, LPS, PHA and

Table 1. The effect of mitogens on the proliferation of sIg^+ or sIg^- cells from HC-primed tilapia

Mitogens	sIg^+	sIg^-
LPS	+++*	+/-
PHA	+/-	+++
Con A	+/-	++

*Hemocyanin-primed tilapia sIg^+ or sIg^- cells (3×10^5/well) were cultured with LPS (50 μg/ml), PHA (5 μg/ml), and Con A (5 μg/ml), respectively. Seventy two hours after incubation, the cells were pulsed with [^{3}H]TdR 8 h before cell harvest. Radioactivity was measured by liquid scintillation spectrometry, and the results were expressed as follows; +/-, weak response (PHA, 4800 ± 730 cpm; Con A, 6200 ± 870 cpm); ++, moderate response (Con A, 64300 ± 7340 cpm); +++, strong response (LPS, 120700 ± 13300 cpm; PHA, 163400 ± 14590 cpm). Error values represent the S.D. from the mean of triplicate wells. The result is sum of three experiments.

Con A. As shown in Table 1, HC-primed sIg^+ cells responded to LPS, whereas sIg^- cells responded very weakly. In contrast, both PHA and Con A were only effective for sIg^- cell proliferation (Table 1). Fig. 1 shows effect of T cell mitogens on the proliferation of sIg^- cells from HC-primed or control tilapia. PHA (5 μg/ml) was more effective in inducing HC-primed T-like cell proliferation than Con A (5 μg/ml) or PHA 0.5 μg/ml (Fig. 1).

Antigen-specific cell proliferation

To identify functional properties of fish T-like cells, the sIg^- and sIg^+ cell populations were incubated in the presence of HC or CA. Unexpectedly, both sIg^+ and sIg^- cell populations showed normal proliferation in the presence of HC, but not of CA (Fig. 2).

NK-like cell cytotoxicity

Chromium 51 release assay was performed to determine whether NK-like cells are also present in fish lymphocytes. Fig. 3 shows cytotoxicity of whole kidney

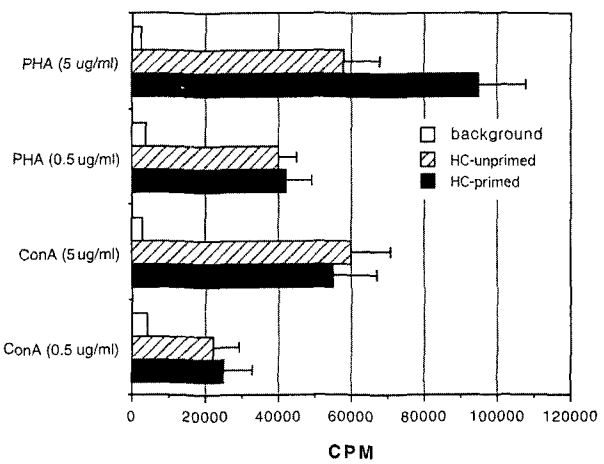

Fig. 1. The effect of T cell mitogens on the proliferation of sIg^- cells from HC-immunized or control tilapia head kidney. The sIg^- cells (1×10^6 cells/well) were cultured for 72 h in 5% CO_2 at 25℃. Cultures were pulsed with 0.5 μCi[^{3}H]-thymidine for 8 h before cell harvest. Radioactivity was measured by liquid scintillation spectrometry, and the results are expressed as cpm incorporation (mean ± S.D.) of triplicates. HC: hemocyanin. The results are representatives of three experiments.

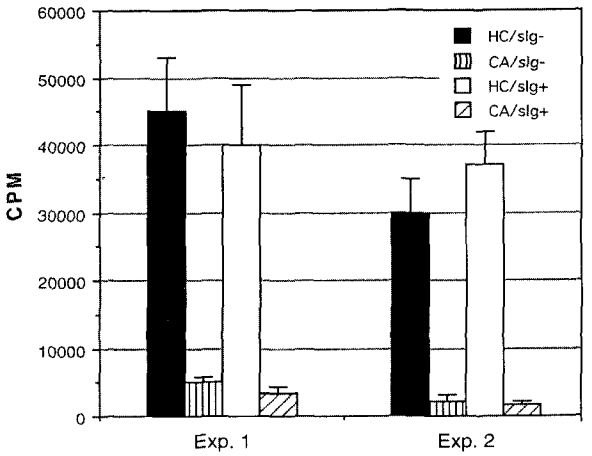

Fig. 2. The proliferation of sIg^+ or sIg^- cells in the presence of HC or CA. Aliquots of 100 μl culture medium containing 2.5×10^5 of HC primed sIg^+ or sIg^- cells were added to 96-well culture plates in the presence or absence of HC. The gamma-irradiated (2000 rad) fish kidney cells (1×10^5 cells/well) were added and followed by 72 h incubation. Cultures were pulsed with 0.5 μCi [^{3}H]-thymidine for 8 h before cell harvest. Radioactivity was measured by liquid scintillation spectrometry, and the results are expressed as cpm incorporation (mean ± S.D.) of triplicates. HC, hemocyanin; CA, chicken albumin.

lymphocytes, which were separated on the Ficoll-Hypaque density gradient, from HC-primed or negative control carp. Lymphocytes from tilapia kidney killed K562 target cells, indicating that NK-like cells exist in fish (Fig. 3). Further, regardless of HC-immunization, fish lymphocytes did not show any difference in cytotoxicity against the target cells (Fig. 3). We also investigated whether NK-like cells in fish can be categorized as sIg^+ or sIg^- cell population. As shown in Fig. 4, NK-like cell cytotoxicity was found in sIg^- cells, suggesting that fish NK-like cells do not bear Ig molecules on their surface and are probably similar to conventional mammalian NK cells.

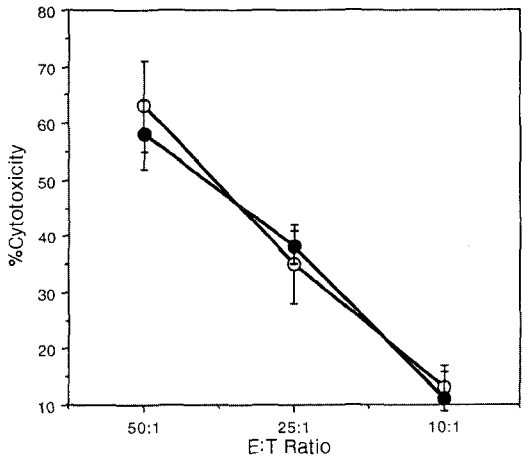

Fig. 3. The cytotoxicity of whole kidney cells from HC-immunized(○) or control tilapia(●). K562 target cell line was used for assays of NK cell cytotoxicity. Effector cells were added (100 μl/well) to each well of 96-well flat-bottom plate with target cell suspension containing 2×10^3 target cells and to different E/T ratios in triplicate. The cell mixtures were incubated for 5 h at 37℃ in a 5% CO_2 condition. The plates were centrifuged and radioactivity of ^{51}Cr in supernatant was measured by γ-spectrometry. Error bar represent the S.D. from the mean of triplicate wells. The results are representative of three experiments.

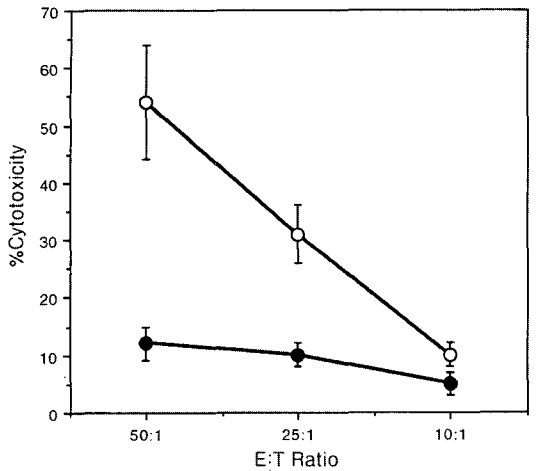

Fig. 4. The cytotoxicity of both sIg^+(●) and sIg^-(○) cells isolated from HC-unprimed tilapia head kidney. As effectors, sIg^+ and sIg^- cells (100 μl /well) from unprimed tilapia head kidney were added to each well of 96-well flat-bottom plate containing 2×10^3 target cells. The cell mixtures were incubated for 5 h at 37℃ in a 5% CO_2 condition. The plates were centrifuged and radioactivity of ^{51}Cr in supernatant was measured by γ -spectrometry. Error bar represent the S.D. from the mean of triplicate wells. The results are representative of two experiments.

Discussion

We have previously reported that the purified sIg^+ cells from BSA-primed carp head kidney showed a humoral immune activity (Choi, 1997) In mammals, it has been clarified that sIg^+ B lymphocytes cannot play their role without the help of T lymphocytes. In the present study, we tried to determine whether T cells also exist in fish as well, and whether the fish T-like cells show any difference in function from conventional T cells. Unfortunately, however, it was almost impossible to purify T cells based on the presently available cell separation system due to the absence of specific monoclonal antibodies against a unique fish T-cell marker. Thus, negatively separated sIg^- cells were used to identify indirectly the properties of fish T-like cells. Since the cellular immune properties on both sIg^+ and sIg^- cell populations were not characterized, we investigated the effect of mitogen, antigen-specific proliferation, and NK-like cell cytotoxicity on each purified cell population. The panning method has been shown previously to be highly effective in separating rainbow trout sIg^+ and sIg^- PBL (Graham and Secombes, 1990b). In our study, however, the cell separation system using Mini-MACS appeared to be more efficient than the panning technique, as demonstrated by FACS analysis (data not shown). The marked dichotomy of proliferation responses was confirmed with sIg^- cells proliferating in response to PHA, with only a very weak response to LPS, and vice versa for the sIg^+ cell population. Indeed, the weak residual proliferation to the opposite mitogen was probably due to the low level of contamination (< 5%) with the opposite population following the Mini-MACS cell separation system. The present proliferation results are in contrast to those of DeLuca et al. (1983) who produced monoclonal mouse anti-trout Ig antibody (I-14) to separate trout head kidney leukocytes by an indirect panning technique. They found that while panning effectively depleted the sIg^+ cells from the non-adherent population, a significant response of these cells to LPS remained. The response to Con A was also present in the adherent population due to the high level contamination with sIg^- cells in their assay. A similar indirect panning technique was used by Sizemore et al. (1984) to separate channel catfish, *Ictalurus punctatus*, PBL into sIg^+ and sIg^- population. Several possible explanations were suggested in both studies, such as incomplete removal of sIg^+ cells, or the presence of LPS-responsive cells in the sIg^- populations. Whatever the reason for such results is, in the present study the responsiveness of the separated sIg^+ and sIg^- PBL populations was functionally distinct. Furthermore, it is certain that the T-like cells of fish function like T cells present in mammals.

Marsden et al. (1995) have reported that the responsiveness of the different PBL populations (unfractionated, sIg^+ and sIg^- cells) from both control fish and fish primed with an A-layer negative strain of *Aeromonas salmonicida* (MT004) grown under iron- restricted conditions demonstrated clear proliferative responses to the *A. salmonicida* antigens. The magnitude of these response in their study was shown to be dose-dependent in both primed and unprimed cells, as seen using unfractionated head kidney cells from MT004 primed trout with whole (formalin-killed) MT004 added *in vitro* (Marsden et al., 1995) and from A-layer positive primed Atlantic salmon, *Salmo salar* (Erdal and Reitan, 1992). Our study shows that there is a strong specific response to HC, but not to the unrelated antigen CA. Thus, we confirmed the presence of antigen specific primed cells in tilapia which are qualitatively similar to those demonstrated by others in trout. However, considering the unexpected normal response of sIg^+ cell population to HC, we cannot exclude the following possibilities: presence of other cell populations in sIg^+ cells participating in antigen- specific response; serious contamination of sIg^- cells; or experimental errors. To further substantiate the result, therefore, more work will have to be performed.

Teleost fishes have lymphoid cells which, based upon functional features, can be considered as B cells, helper T cells, and accessory cells with reasonable confidence (Clem et al., 1991). However the identification of various effector cells (i.e. cytotoxic T cells, NK cells, ADCC cells) with cytotoxic activities in teleosts is not so clear. The best studied system in this regard involves NK-like cells, termed natural cytotoxic cells (NCC), in the channel catfish. Catfish NCC abound in the pronephron (but not blood) and exhibit significant cytotoxicity for a wide variety of xenogeneic targets (Graves et al., 1984). In our study, irrespective of priming, no different level of NK-like cell

cytotoxicity was observed in the head kidney leukocytes. In this regard, we can speculate that HC antigen itself is not a good candidate for activating NK-like cells since their activities would be induced against only virally infected or abnormally growing target cells. In another experiment, we found that NK-like cell cytotoxicity was observed only in sIg^- cell population. The observations reported herein appear to strongly support the notion that the cytotoxic effector cells are present in catfish peripheral blood as well as in the pronephron and function like mammalian NK cells.

In summary, our studies demonstrate that some T-like cell population in sIg^- cells from tilapia kidney function like conventional mammalian T cells and NK-like cells. However, further studies should be done to find out the reason why even sIg^+ cells from tilapia kidney showed the antigen specific response. In addition, in order to investigate, in addition, the exact role of each cell population in fish leukocytes, various monoclonal antibodies against cell surface markers should be developed. Better understanding of functional fish lymphocytes will be helpful in evaluating and developing the effective vaccines for fishes, and monitoring the course of therapy in infected fishes.

Acknowledgements

The authors gratefully acknowledge financial support for this work by grant of Woosuk University research fund in 2000.

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[Received September 2, 2000; accepted October 30, 2000]