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Influence of Temperature Shifts on Antibody Synthesis in the Oliver Flounder (*Paralichthys olivaceus*) Immunised with Formalin Killed *Edwardsiella tarda* Antigen

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The effects of various temperature shifts on the kinetics of the humoral antibody response in oliver flounder, *Paralichthys olivaceus*, immunised with formalin-killed *Edwardsiella tarda*, were determined by measuring the antibody production *in vivo* and *in vitro*. When fish acclimated to a high temperature and immunised at that temperature were transferred to a lower temperature (22℃ to 12℃) at a various times after immunisation, the fish showed a weaker immune response than that achieved when the fish were kept at a high environmental temperature. However, in the converse experiment (12℃ to 22℃), the magnitude of the humoral immune response was recovered independent of the time of the transfer after immunisation at low temperature, even though the peak levels of each transferred group did not reach the level found in the positive control group that was maintained and immunised at a high environmental temperature. Hence, these studies provide some evidence that the potential for antibody production in B cells of oliver flounder immunized at high temperature is not impaired by subsequent exposure to low temperature.

Key words: Immune response, Antibody production, Temperature shifting, B cell, Oliver flounder.

Introduction

Mechanism of low temperature-induced immunosuppression in fish has been the focus of considerable research because of its economic importance for the aquaculture industry (Rijkers *et al*., 1980; Miller and Clem, 1984; Waterstrat *et al*., 1991; Alcorn *et al*., 2002). However, little is known about the effect of different environmental temperatures within the physiological range on the immune responses of ectothermic vertebrates, except for the fact that higher temperature causes faster and higher-magnitude antibody production (Secombes *et al*., 1991; Watts *et al*., 2001; Nath *et al*., 2006). Furthermore, few data are available comparing the specific effects of 'shift up' and 'shift down' of environmental temperatures during immune reaction *in vivo* (Bly and Clem, 1992; Attia-El-Hili *et al*., 1992). Consequently, the effects of temperature decreases and increases on the expression of specific immunity needs further study. Additionally, the kinetics of the effects of temperature shifts on different stages of the immune response need to be studied since many previous reports do not contain this information.

The aim of the present study was to determine the effects of environmental temperature changes during the *in vivo* immune reaction on antibody production after immunisation with formalin-killed bacteria in oliver flounder. Furthermore, to deter-

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mine the mechanisms by which temperature modulates the immune system of oliver flounder, we evaluated the kinetics of the immune responses by shifting the environmental temperature at various time points and confirmed the results by *in vitro* experiments.

Materials and Methods

Fish and bacteria

Oliver flounder (*Paralichthys olivaceus*), mean weight 100 g, were obtained from a farm in the East Sea area of Korea. Fish were acclimated to the desired temperature for a period of at least 3 weeks before use in 800 ℓ circular tanks at 12℃ or 22℃. *E. tarda* 219 strain, isolated in my laboratory from oliver flounder suffering from edwardsiellosis, was used.

Antigen preparation

Formalin-killed cells (FKC) and an EDTA-released outer membrane complex (EDTA-Ag) were used as antigens for the immunisation of oliver flounder. *E. tarda* 219 was incubated, with shaking, in Tryptic Soy Broth (Difco, Detroit, MI, USA) at 25℃ for 24 h, which produced 9×10^9 cells/㎖. The cells then were killed with 0.5% formalin and washed three times with PBS before use. For EDTA-Ag, bacteria that had been cultured overnight were harvested by centrifugation at 6,000 ×g for 15 min and washed twice with PBS, pH 7.2. The bacterial pellet was resuspended in PBS containing 20 mM EDTA (10^{-1} volume of cultured broth) and incubated at 45℃ for 30 min with gentle agitation. After sonication for 60 sec (10% power level with medium tip, Vibra Cell sonication, 375 W, USA), the bacterial suspension was centrifuged at 6,000 ×g for 30 min to remove the cell debris. The supernatant containing the EDTA-Ag was dialysed against PBS (cut out 5000 dalton). The concentration was determined by measuring the absorbance at 280 nm.

Antiserum production

(a) Oliver flounder immunoglobulin

Antiserum obtained from oliver flounder immunised twice with formalin-killed *E. tarda* 219 was precipitated with 25% w/v saturated ammonium sulfate. After centrifugation, the supernatant was reprecipitated with 50% w/v saturated ammonium sulfate. The precipitated pellet was resuspended in PBS pH 7.2 at a concentration of 10 ㎎/㎖. Purified immunoglobulin of oliver flounder was prepared by chromatography on a DEAE-5PW column in HPLC (TOSCH, Tokyo, Japan), monitoring the effluent by a test of the ability of each fraction to cause the agglutination of formalin-killed *E. tarda* 219.

(b) Rabbit anti-oliver flounder Ig sera

The purified Ig fraction from oliver flounder serum was mixed 1:1 with Freund's complete adjuvant (FCA) and 1 ㎖ (200 protein/㎖) was subcutaneously injected into a New Zealand White rabbit (Hyochang Science, Taegu, South Korea). At 2-week intervals, 3 boosters were administered by injection of the immunogen mixed 1:1 with Freund's incomplete adjuvant (FIA). The final bleeding was done 1 week after the last immunisation. The IgG fractions of the rabbit antiserum were isolated by precipitation with ammonium sulfate followed by a protein-A column (FPLC, Pharmacia, Uppsala, Sweden). For biotin labelling, biotinamidocaproate N-hydroxysuccinimide (Sigma Chemical Co. Ltd.) was dissolved in dimethylsulfoxide (2 ㎎/㎖) and 25 ㎕ of this solution was added to 1 ㎎ of the purified rabbit IgG fractions (1 ㎎/㎖ $NaHCO_3$ buffer, pH 8.3). The mixture was incubated for 3 h at 37℃

and dialyzed overnight against PBS containing 0.05% sodium azide.

Shifting of environmental temperature

Experimental fish were immunised with a single i.p injection of 2 ㎎ (wet weight) of FKC emulsified in FCA. Immediately afterward and 1, 2, and 4 weeks after immunization at 12℃ or 22℃, fish were transferred to water at a temperature of 22℃ or 12℃. The immune response was studied by examining 5 fish at 1, 2, 3, and 5 weeks after the shift of environmental temperature.

Agglutinating antibody assay

Antibody titres against *E. tarda* 219 strain were determined by agglutination tests in 96-well microtitre plates (Sigma) as described by Roberson (1990). Samples (50 ㎕ each) were serially two fold diluted in PBS and 50 ㎕ FKC *E. tarda* 219 (3 ㎎/㎖) was added to each well and thoroughly mixed. Plates were incubated at room temperature overnight prior to examination for agglutination. The antibody titres were expressed as the highest sample dilution giving positive agglutination.

In vitro antibody production

At varying times after immunisation or shifting environmental temperatures, fish were killed and erythrocyte-free spleen cell suspensions were prepared by separation on a 51% Percoll (Sigma) gradient and diluted in L-15 medium (Sigma) containing 100 ㎍/㎖ penicillin and 100 units/㎖ streptomycin and 5% fetal calf serum (FCS, Sigma).

Cells were adjusted to two different densities (10^7 and 10^6 cells/well) and were incubated and allowed to secrete antibodies in the 96-well plate for 24 h at 22℃ in a 5% CO_2 incubator. After incubation the supernatant of each well was collected and used for an ELISA to determine the concentration of secreted antibody. Briefly, the supernatants (50 ㎕) were added to wells coated with 100 ㎕/well of EDTA-Ag at 50 ㎍/㎖ in PBS and incubated for 1.5 h. After washing three times with PBS containing 0.1% Tween 20 (PBS-T) and then blocking the unbound sites with 2% bovine serum albumin (BSA), biotin-conjugated rabbit anti-oliver flounder Ig (1 ㎎/㎖), diluted 1:40 in 0.2% BSA/PBS was added to each well and incubated at 37℃ for 40 min. Extravidin-conjugated alkaline phosphatase (Sigma) diluted 1:5,000 in 0.2% BSA/PBS was added and the plates were incubated for 40 min. After three washes the substrate, p-nitrophenyl phosphate (Sigma) in tris buffer (pH 9.8), was added to the wells. The absorbancies were read with a Bio-kinetics reader (Bio-Tek, Winooski, VT, USA) at 405 nm. A discrimination index (DI) was determined by comparison of the absorbancies of the experimental samples and the negative control, which tested the isolated leukocytes from unimmunised fish.

$$DI = \frac{\text{Average absorbance of experimental sample-Average absorbance of the negative control}}{\text{Average absorbance of the negative control}} \times 100\ (\%)$$

Results

Serum antibodies

To determine the effects of water temperature shift on antibody production during the immune reaction, 12℃-acclimated oliver flounders were immunised with 20 ㎎/㎏ body weight of *E. tarda* FKC as an antigen at the same water temperature. After various time periods, immediately after immunization, 1, 2 and 4 weeks later, oliver flounders of each group were transferred to a tank with a water temperature of 22℃. Blood samples for antibody production assays were drawn at 1, 2, 3, and 5 weeks following transfer.

The results obtained with the five oliver flounders tested are given in Fig. 1. The levels of anti *E. tarda*

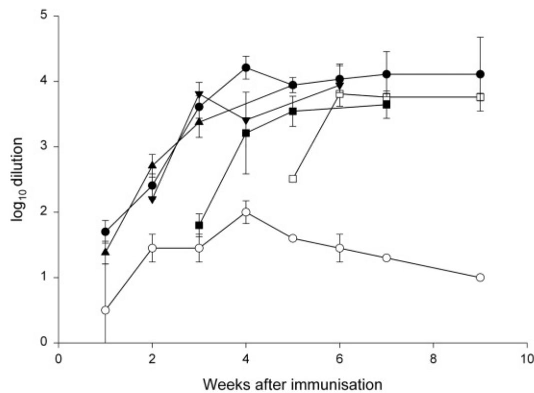

Fig. 1. The effects of shifted temperature on the kinetics and amounts of specific antibody in serum following immunisation of fish. 12℃-acclimated fish were transferred to water at a temperature of 22℃ at various time points after immunization. Data are expressed as the mean of 4-6 fish. ▲, transferred to 22℃ immediately after immunisation; ▼, transferred to 22℃ at 1 week after immunisation; ■, transferred to 22℃ at 2 weeks after immunisation; □, transferred to 22℃ at 4 weeks after immunisation; ●, acclimated and immunised at 22℃ without disturbance (positive control); ○, acclimated and immunised at 12℃ without disturbance (negative control).

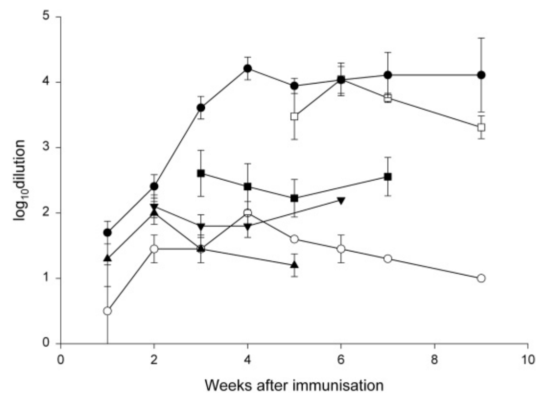

Fig. 2. The effects of shifted temperature on the kinetics and amounts of specific antibody in serum following immunisation of fish. 22℃-acclimated fish were transferred to water at a temperature of 12℃ at various time points after immunization. Data are expressed as the mean of 4-6 fish. ▲, transferred to 12℃ immediately after immunisation; ▼, transferred to 12℃ at 1 week after immunisation; ■, transferred to 12℃ at 2 weeks after immunisation; □, transferred to 12℃ at 4 weeks after immunisation; ●, acclimated and immunised at 22℃ without disturbance (positive control); ○, acclimated and immunised at 12℃ without disturbance (negative control).

antibody in serum of the oliver flounder transferred to a high environmental temperature immediately after immunisation increased for 5 weeks and reached a level similar to that of the positive control (maintained at 22℃ without temperature shifting after immunisation). However, the oliver flounders transferred to a high water temperature at week 1, 2, or 4 post-immunisation showed an increase of specific antibody for 2 weeks and then the antibody levels remained constant. The peak levels of these groups were similar to each other and did not reach those of the positive controls.

In the converse experiment, the 'shift down' temperature protocol (Fig. 2), the antibody concentration in the oliver flounders transferred to a low environmental temperature immediately after immunisation at a high temperature showed no significant differences compared to the negative control (maintained at 12℃ without temperature shifting after immunisation).

However, an increased level of the antibody production was observed in the groups of oliver flounder exposed to antigen for more than 1 week at 22 ℃ before being transferred to 12℃. Interestingly, the antibody concentrations in these groups remained constant throughout the experimental periods at low water temperature.

Antibody production *in vitro*

Leukocytes isolated from fish immunised at 22℃ and transferred immediately or after 1 week to 12℃ were not able to produce antibody *in vitro* (Fig. 3).

However, significant antibody production was detectable in supernatant from leukocytes isolated from fish immunised at 22℃ for 2 or 4 weeks prior to the 'shift down' of environmental temperature. In particular, leukocytes isolated from the latter group produced almost the same levels of antibody as was

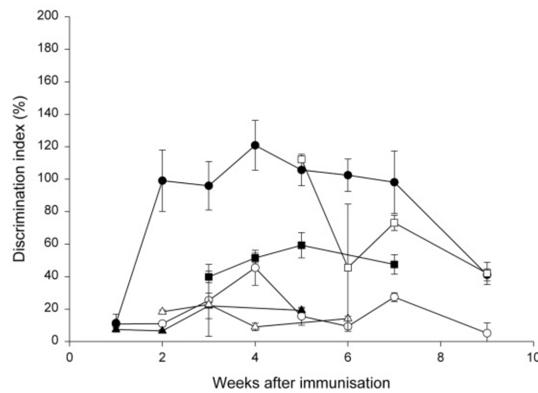

Fig. 3. The kinetics and amounts of *in vitro* antibody production using the isolated leukocytes from the fish influenced by shifted environmental water temperatures during immune reaction. 22℃–acclimated and immunised fish were transferred to 12℃ water temperature at various time points during the immune reaction. Isolated leukocytes in the spleen were cultured at a concentration of 10^6/well in L-15 containing 5% FCS for 24h at 22℃ before the collection of supernatant for analysis. Data are expressed as the mean of D.I. ± S.E. obtained with an ELISA of 3 fish. ▲, transferred to 12℃ immediately after immunisation; △, transferred to 12℃ at 1 week after immunisation; ■, transferred to 12℃ at 2 weeks after immunisation; □, transferred to 12 ℃ at 4 weeks after immunisation; ●, acclimated and immunised at 22℃ without disturbance (positive control); ○, acclimated and immunised at 12℃ without disturbance (negative control).

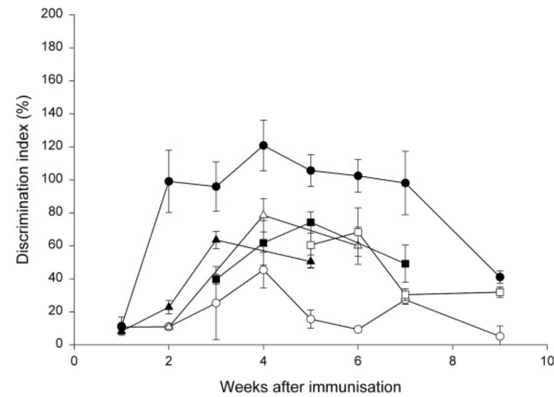

Fig. 4. The kinetics and magnitudes of *in vitro* antibody production using the isolated leukocytes from the fish influenced by shifted environmental water temperatures during immune reaction. 12℃–acclimated and immunised fish were transferred to 22℃ water temperature at various time points during immune reaction. Isolated leukocytes in the spleen were cultured at a concentration of 10^6/well in L-15 containing 5% FCS for 24 h at 22℃ before the collection of supernatant for analysis. Data are expressed as the mean of D.I. ± S.E. obtained with ELISA of 3 fish. ▲, transferred to 22℃ immediately after immunisation; △, transferred to 22℃ at 1 week after immunisation; ■, transferred to 22℃ at 2 weeks after immunisation; □, transferred to 22℃ at 4 weeks after immunisation; ●, acclimated and immunised at 22℃ without disturbance (positive control); ○, acclimated and immunised at 12℃ without disturbance (negative control)

found in the positive control group.

In 'shift up' temperature protocols, the leukocytes isolated from the oliver flounder transferred from 12℃ and maintained at 22℃ for more than 1 or 2 weeks were able to produce a much greater amount of antibody compared to the negative controls and reached the peak level at a relatively rapid rate (Fig. 4). We also confirmed that the kinetics of antibody production *in vitro* with isolated leukocytes were similar to those of *in vivo* analysis with the serum of fish (Figs. 1 and 2).

Discussion

The effect of changing environmental temperatures therefore could have an adverse impact on the aquaculture industry as a consequence of its effect on the immune response (Pylkko *et al*., 2002). However, few data are available with regard to the detailed influence of environmental temperature on immune expression of oliver flounder, the main fish species of marine aquaculture for more than 10 years in Asian countries including Korea. Thus, we have assessed the effects of environmental temperatures, both higher and lower during immune reactions in oliver flounder. Two temperatures were used; one the optimum temperature (22℃) and the other lower (12℃) but within the physiological temperature range for growth of oliver flounder.

The present result showed that antibody produc-

tion of oliver flounder leukocytes was a temperature-dependent phenomenon both *in vivo* and *in vitro.* Antibody concentrations in serum collected from fish subjected to a temperature shift from 22 ℃ to 12℃ at 4 different time points after immunisation were stable for 5 weeks at the levels achieved at a high temperature before transfer (Fig. 2). In the converse experiment wherein fish were immunised at 12℃ then transferred to 22℃, the production of antibody was restored rapidly and independently of the time after immunisation at which the fish were transferred (Fig. 1). Moreover, *in vitro* cultured leukocytes isolated from fish immunised at higher or lower temperature and then transferred to a lower or higher temperature various periods after immunisation also synthesized and released immunoglobulin into the medium according to the same patterns found *in vivo* in blood (Figs. 3 and 4). Thus, the kinetics of the immune responses both in *in vivo* and *in vitro* experiments suggested that a shift-down of environmental temperature blocked further production of antibody but that a shift-up reversed the inhibitory effect.

Even though it is generally accepted that the effects of temperature on immune reactions are due mainly to the sensitivity of T cells and possibly of B cells, or the hemeoviscous adaptation of their plasma membranes (Bly and Clem, 1988; Engelsma *et al*., 2003), it would be necessary to determine the relationships of these events by direct comparison of the *in vivo* kinetics of immune responses on higher and lower environmental temperatures rather than in an *in vitro* analysis with cultured leukocytes.

Bly and Clem (1991) have showed that the abrupt dropping of the water temperature to 12℃ for channel catfish acclimated at 23℃ almost completely inhibited the responses of B cell and T cells to mitogens, their responses in mixed leukocyte reactions, and their antibody production in response to antigens.

The suppression of antibody production in oliver flounder (Figs. 2 and 3) acclimated and immunised at 22℃, and transferred to 12℃ agreed with the results obtained with the *in vitro* immune responses of peripheral blood leukocytes taken from channel catfish (Bly and Clem, 1991).

However, it only partially agreed with the results of the study of the grass carp, which showed the suppressive influence of low environmental temperatures only on the induction phase or early stages of the immune reaction (Yang and Zuo, 1991). Thus, as long as grass carp has been at the critical temperature within 5 days after being immunised with antigen, the synthesis and release of antibody rise after transfer to the optimum water temperature. In the present study, it appeared that B cells that had already differentiated to antibody-producing cells at the higher water temperature after immunisation still secreted antibody in serum or *in vitro* cultured medium after a shift to lower water temperature for more than 5 weeks without a change from the levels that had been achieved at the higher water temperature (Figs. 2 and 3).

Further research to reveal the effects of temperature on the immune systems of ectothermic vertebrates is needed to obviate the detrimental effects of low water temperature on industrial aquaculture.

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References

Alcorn, S. W., Murra, A. L. and Pascho, R. J.: Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). Fish Shellfish Immunol., 12: 303-

334, 2002.

Attia-El-Hili, H., El-Gaaid, A., Troutaud, D. and Deschaux, P.: Study of reared sea bream antibody response to a sheep red blood cells injection. Ichtyophysiol. Acta, 15: 7-20, 1992.

Bly, J. E. and Clem, L. W.: Temperature-mediated processes in teleost immunity : Hemeoviscous adaptation by channel catfish peripheral blood cells. Comp. Biochem. Physiol., 914: 481-485, 1988.

Bly, J. E. and Clem, L. W.: Temperature-mediated processes in teleost immunity : *in vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. Vet. Immunol. Immunopathol., 28: 365-377, 1991.

Bly, J. E. and Clem, L. W.: Temperature and teleost immune functions. Fish Shellfish Immunol., 2: 159-171, 1992.

Engelsma, M. Y., Hougee, S., Nap, D., Hofenk, M., Rombout, J. H., van Muiswinkel, W. B. and Verburg-van Kemenade, B. M. L.: Multiple acute temperature stress affects leucocyte populations and antibody responses in common carp, *Cyprinus carpio L.* Fish Shellfish Immunol., 15: 397-410, 2003.

Miller, N. W. and Clem, L. W.: Temperature-mediated processes in teleost immunity : differential effects of temperature on catfish *in vitro* antibody responses to TD and TI antigens. J. Immunol., 133: 2356-2359, 1984.

Nath, S., Kales, S., Fujiki, K. and Dixon, B.: Major histocompatibility class II genes in rainbow trout (*Oncorhynchus mykiss*) exhibit temperature dependent downregulation. Immunogenetics, 58: 443-453, 2006.

Pylkko, P., Lyytikainen, T., Ritola, O., Pelkonen, S. and Valtonen, E. T.: Temperature effect on the immune defense functions of Arctic charr Salvelinus alpinus. Dis. Aquat. Org., 52: 47-55, 2002.

Rijkers, G. T., Frederix-Wolters, E. M. H. and Van Muinswinkel, W. B.: The immune system of cyprinid fish. Kinetics and temperature dependence of antibody producing cells in carp (*Cyprinus carpio*). Immunol., 41: 91-97, 1980.

Secombes, C. J., White, A., Fletcher, T. C. and Houlihan, D. F.: The development of an ELISPOT assay to quantify total and specific antibody secreting cells in dab *Limanda limanda* (L). Fish Shellfish Immunol., 1: 87-89, 1991.

Waterstrat, P. R., Ainsworth, A. J. and Capley, G.: In vitro responses of channel catfish (*Ictalurus punctatus*) neutrophils to *Edwardsiella ictaluri*. Dev. Comp. Immunol., 15: 53-63, 1991.

Watts, M., Munday, B. L. and Burke C. M.: Immune responses of teleost fish. Aust. Vet. J., 79: 570-574, 2001.

Yang, X. and Zuo, W.: Mechanism of water temperature-dependent immune response in grass carp (*Ctenopharyngodon idella*), J. Chin. Acad. Fish. Sci. Zhongguo Shuichan Kexue Yanjiuyuan Xuebao., 4: 27-33, 1991.

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