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Influence of temperature shifts on the kinetics of the specific antibody secreting cells in the olive flounder *Paralichthys olivaceus*

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We determined the effects of temperature shifts on the kinetics of the numbers of antibody-secreting cell (ASC) in the olive flounder *Paralichthys olivaceus* immunised with formalin-killed *Edwardsiella tarda*. When fish that were acclimated to 22℃ and immunised at that temperature were transferred to a lower temperature (12℃) at a various times (immediately, 1, 2 or 4 weeks) after immunisation, both further differentiation of B cells and secretion of antibody from the ASC developed at 22℃ were suppressed at 12℃. 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 22℃ control group. The results were confirmed by counting the number of specific antibody secreting cells (SASC) in the spleen. This study provides the evidences of the immune reaction that the potential for antibody production in B cells of flounder, the most important species in aquatic industry of Korea, immunized at high temperature is suppressed by subsequent exposure to low temperature and that low temperature-induced humoral immuno suppression can be reversed by exposure to a higher temperature.

Key words: Temperature, Flounder, Immune response, Antibody secreting cells

Various leukocyte subpopulations, including T cells (Miller *et al*., 1986), B cells, and accessory cells (Bly and Clem, 1992 Hurubec *et al*., 1996; Bromage *et al*., 2004), as well as diverse effector molecules such as interleukins (Ellsaesser *et al*., 1988; Lee *et al*., 2006) are involved co-operatively in the establishment of the adaptive immune responses in endothermic vertebrates. Responses similar to those in endothermic vertebrates are also occurring in ectothermic vertebrates (Corbel, 1975; Denzin and Staak, 2000; Tort *et al*., 2003; Wilson and Warr, 1992). Moreover, such responses in ectothermic vertebrates would be expected to occur over a wide range of environmental temperatures in order to adequately protect against infectious diseases.

The 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; Waterstrat *et al*., 1991; King *et al*., 2006; Hawley *et al*., 2008). However, little is known about the effect of different environmental temperatures within the physiological range on the immune

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responses of ectothermic vertebrates, except for the fact that higher temperature causes faster and higher-magnitude antibody production (Morvan *et al.*, 1998; Secombes *et al.*, 1991). Furthermore, few data are available comparing the specific effects of 'shift up' and 'shift down' of environmental temperatures during immune reaction in vivo (Attia *et al.*, 1992; Bly and Clem, 1992). Consequently, the effect of temperature variation on the expression of specific immunity needs further study, especially the effects on the antibody-producing cells that resulted from antigen stimulation before the occurrence of the temperature shift. Moreover, worth while study, 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 on the production of antibodies in olive flounder (*Paralichthys olivaceus*) immunized with formalin-killed bacteria.

Materials and Methods

Fish and bacteria

Olive flounder, 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 our laboratory from olive 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 flounder and for the detection of specific antibody-secreting cells in the Enzyme-Linked Immuno spot (ELISPOT) assay, respectively. *E. tarda* 219 was incubated, with shaking, in Tryptic Soy Broth (Difco, Detroit, MI, USA) at 25℃ for 24 h, which produced 9 x 10^9 cells/ml. The cells then were killed with 0.5% formalin and washed three times with PBS(pH 7.2) 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. 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 cells. The supernatant containing the EDTA-Ag was dialysed against PBS. The concentration was determined by measuring the absorbance at 280 nm.

Antiserum production

(a) Flounder immunoglobulin. Antiserum obtained from olive 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 at a concentration of 10 ㎎/㎖. Purified immunoglobulin of 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-flounder Ig sera. The purified Ig fraction from flounder serum was mixed 1:1 with

Freund's complete adjuvant(FCA) and 1 ㎖ (200 ㎍ protein/㎖) was injected into a New Zealand White rabbit subcutaneously. At 2-week intervals, 3 times boosters were administered by injection of the immunogen mixed 1:1 with Freund's incomplete adjuvant. 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 ㎎ 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 4-6 fish at different time after the shift of environmental temperatures.

ELISPOT assay

The ELISPOT assay was used for quantifying SASC. Nitrocellulose-bottom, 96-well microplates were coated for 3.5 h at 37℃ with EDTA-Ag (50 ㎍/㎖ PBS) at 100 ㎕/well. The plates were always placed in a humidified atmosphere. After washing three times with PBS-T (0.05% Tween 20 in PBS), unbound sites on the plates were blocked by the addition of 2% bovine serum albumin (BSA) in PBS-T for 1 h at 37℃. The wells were washed twice with PBS-T and once with PBS before adding the cells. Isolated splenic leukocytes were allowed to secrete antibodies in the CO_2 incubator (CO_2 5%) for 6 h at 22℃. Three different cell densities were used in triplicate: 1 x 10^6, 1 x 10^5, and 1 x 10^4 cells/well. Biotin-conjugated rabbit anti-flounder Ig (1 ㎎/㎖), diluted 1:40 in PBS containing 0.2% BSA, were added to each well and incubated overnight at 4℃. After washing, extravidin-conjugated alkaline phosphatase (Sigma, U.S.A) diluted 1:1,000 in PBS containing 0.2% BSA was added and the plate was incubated at 37℃ for 1 h. This was followed by three washes in PBS-T and the addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma, U.S.A). Plates were allowed to develop for 10 min at room temperature, and then washed in distilled water. Finally, plates were observed using a light stereomicroscope. Only the highest concentration giving an approximate spot density of 50-400 spots was used to count and calculate the results. Two controls were run for each sample of cells, one on EDTA-Ag uncoated wells and the other one treated with cycloheximide (100 ㎍/㎖) on ice for 2 hrs, and these were always negative.

Results

The kinetics of antibody-secreting cells in 'shift down' temperature

The numbers of SASC in the leukocytes isolated from the spleens of flounder were counted. The peak SASC response in flounder of the positive control group could be detected at week 4 post-immunisation, after which there was a continuous decrease to levels that were almost undetectable by week 9. The peak SASC response at week 4 was

dramatically enhanced in the positive control group of 22°C as compared with that seen in the negative control group of 12°C (Fig. 1).

Moreover, shifting the environmental temperature down immediately or one week after immunization completely suppressed the appearance of SASC to

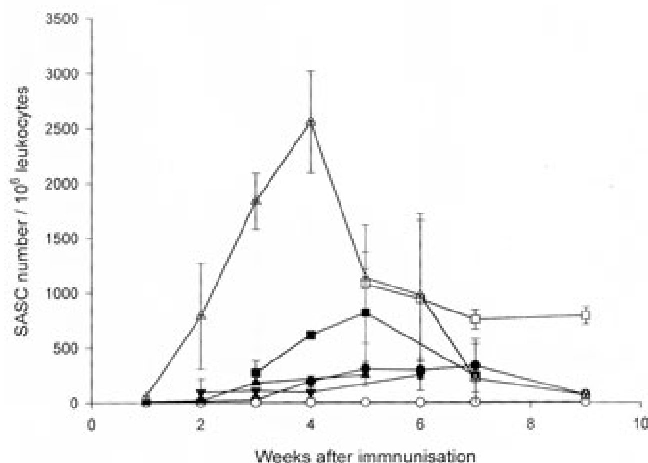

Fig. 1. The effects of shifted temperature on the kinetics and magnitudes of SASC responses in the spleen following immunisation of fish with *E. tarda* 219 FKC. 22°C-acclimated fish were transferred to 12°C water temperature at various time points after immunization. Data are expressed as the mean ±S.E. of 4-6 fish. ▲: transferred to 12°C immediately after immunisation; ▼: transferred to 12°C at 1 week after immunisation; ■: transferred to 12°C at 2 weeks after immunisation; □: transferred to 12°C at 4 weeks after immunisation; △: acclimated and immunised at 22°C without disturbance (positive control); ●: acclimated and immunised at 12°C without disturbance (negative control); ○: unimmunized control at 22°C.

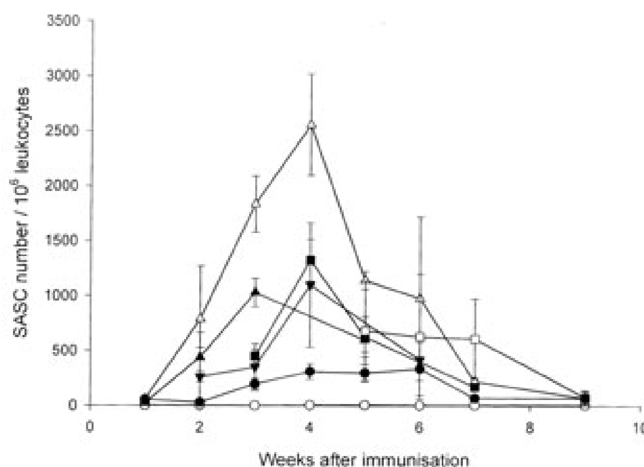

Fig. 2. The effects of shifted temperature on the kinetics and magnitudes of SASC responses in the spleen following immunisation of fish with *E. tarda* 219 FKC. 12°C-acclimated fish were transferred to 22°C water temperature at various time points after immunization. Data are expressed as the mean ±S.E. of 4-6 fish. ▲: transferred to 22°C immediately after immunisation; ▼: transferred to 22°C at 1 week after immunisation; ■: transferred to 22°C at 2 week after immunisation; □: transferred to 22°C at 4 week after immunisation; △: acclimated and immunised at 22°C without disturbance (positive control); ●: acclimated and immunised at 12°C without disturbance (negative control); ○: unimmunized control at 22°C.

levels found in the negative control group (Fig. 1). However, the numbers of SASC in fish transferred to 12℃ at week 2 or 4 after immunisation at 22℃ were different from those of fish transferred at an early stage of the immune reaction. The former group, transferred 2 weeks after immunisation, showed slightly and gradually increasing numbers of SASC for 3 weeks and then the numbers decreased to the levels of the negative control. The latter group, transferred 4 weeks after immunisation, showed greater numbers of SASC compared to those of the former and the levels decreased continuously without peaking (Fig.1).

The kinetics of antibody-secreting cells in 'shift up' temperature

The numbers of SASC in fish transferred to 22℃ after immunisation at 12℃ also were determined (Fig. 2). The numbers of SASC among the leukocytes isolated from flounder transferred to 22℃ within less than 2 weeks (immediately, at week 1, and at week 2) post-immunisation at 12℃ began to increase and showed peak numbers at 3-4 weeks after transfer. However, none of these three groups ever reached the peak levels found in the positive control. Flounder transferred to 22℃ at week 4 post-immunization also showed increased numbers of SASC but the numbers continuously decreased throughout the experimental periods without peaking.

Discussion

The entire physiology of the teleost, particularly its immune function, is affected by the environmental temperature (Lankford *et al*., 2003; Vainikka *et al*., 2004; King and Berlinsky, 2006). It is generally accepted that higher environmental temperatures enhance antibody formation, whereas lower temperatures adversely affect ectothermic animals such as fish. Thus, we have assessed the effects of environmental temperatures, one the optimum temperature (22℃) and the other lower (12℃) but within the physiological temperature range for growth of flounder, on the kinetics of B cell differentiation during immune reactions in flounder. It allowed us to examine the relationships between antibody production and shifted environmental temperatures after immunisation with antigen. Thus, in this study, the changed immune-kinetics caused by 'shift up' or 'shift down' of temperatures at different stages of the immune reaction after immunisation would provide some important clues to achieve a better understanding of the phenomenon of temperature-induced immune system effects in the flounder.

The maximum SASC response in flounder kept at 22℃ was observed on day 28 after immunisation (Fig. 1). Immune kinetic studies of cultured flounder using the ELISPOT method have not been reported before, and our results showed slightly postponed immune responses compared to the studies on carp *Cyprius carpio* by Rijkers *et al*., (1980), who found a peak response on day 10-12 after immunisation of fish kept at 20℃. However, roach kept at 18℃ appeared to show the peak response on day 21 post immunisation with bovine gamma globulin (Aaltonen *et al*., 1994). Similarly, we also could not find a more gradual increase in the number of SASC, like the studies of Davidson (1992), after the peak response at week 7-8 post immunization. Like the results of several reported studies, the kinetics of the response in fish is dependent on the temperature as well as on diet, fish species, ages, and possibly antigens (Pourreau *et al*., 1986; Nakanishi, 1987; Koskela *et al*., 2004; Bagni *et al*., 2005).

In the present study, Immune kinetic studies of

the changes in the numbers of ASC suggested that B cells committed to the production of ASC by stimulation at the optimum temperature for more than 2 weeks could mature to ASC even after transfer to a low water temperature (Fig. 1). However, B cells in the flounder stimulated less than 1 week at the optimum water temperature did not developed into ASC after transfer of the fish to a low water temperature. Thus, the effects of low-temperature-induced immunosuppression of B cell antibody secretion or maturation to antibody secretion were not prevented by immunisation at a high temperature.

In 'shift-up' temperature protocols, the increased numbers of ASC in all groups of flounder (Fig. 2) transferred to a high temperature even after 4 weeks of immunisation at a low water temperature implied that T cells were functional in helping B cells even at a low water temperature. In catfish, it was determined that if fish were acclimated to low temperatures, T cell responses were restored and indicated the in vivo generation and activation of virgin T cells even at low temperature (Clem *et al*., 1984). If that were not the case, B cells in fish after 4 weeks of immunisation at low water temperature could not have been committed to forming ASC or antibody-synthesizing cells because of the absence of T cell function or by the disappearance of antigen in vivo on that time point. Additionally, it is important to point out that the T cells were as able as B cells to patch and cap receptor-ligand complexes and the accessory cells were able to effectively process and present antigen at low temperatures.

Results of this study have suggested the commitment of B cells to the formation of antibody-producing cells at low water temperatures in flounder in the 'shift up' water temperature experiment. Also, we have demonstrated that a 'shift down' in temperature of flounder during an immune reaction suppressed the differentiation of B cells but not the secretion of antibody from cells derived from B cells that had been previously activated at high temperature.

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