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SHORT COMMUNICATION

Effect of environmental temperature on *in vitro* cell-mediated cytotoxicity (CMC) and graft-versus-host reaction (GVHR) in ginbuna crucian carp (*Carassius auratus langsdorfii*)

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Fish belong to the poikilothermic vertebrates and vital functions including cellular and humoral immune responses are influenced by environmental temperature. Weiss and Avtalion (1977) first suggested that in carp helper memory maturation is more suppressed at low temperatures than antibody synthesis. In channel catfish, suppression of humoral immunity at low temperature was found to be a result of inhibitory effect on T helper cells rather than B cells (Clem *et al*., 1984; Miller & Clem, 1984; Bly & Clem, 1991; Vallejo *et al*., 1992), and mixed leucocyte reaction as an *in vitro* test for T helper cell function is suppressed at low temperature (Miller *et al*., 1986).

Cell-mediated cytotoxicity (CMC) includes non-specific and specific reactions. Non-specific cytotoxic cells (NCC; Evans & Jaso-Friedmann, 1992), the functional equivalent to mammalian natural killer (NK) cells, are affected by temperature in fish (Kurata *et al*., 1995; LeMorvan-Rocher *et al*., 1995; Pettey & McKinney, 1983). Specific CMC is performed by cytotoxic T cells in higher vertebrates. Cytotoxic T cells recognise other cells mainly by the interaction of their T cell receptor and CD8 molecules with the major histocompatibility complex (MHC) expressed on all cells. If the MHC is incompatible, e.g. in the case of allogeneic cells, or if the peptide expressed together with the MHC is foreign, those cells will undergo killing by specific cytotoxic cells. Specific cell-mediated cytotoxicity against hapten modified syngeneic cells (Verlhac *et al*., 1990) and allograft rejection (Hildemann, 1957; Rijkers, 1982; Nakanishi, 1985) is suppressed at low temperature in fish. However, it has not been demonstrated clearly whether mainly helper cells or cytotoxic cells are suppressed.

Specific CMC against allogeneic fish cells has been shown *in vitro* (Fischer *et al*., 1998*a*). In this *in vitro* assay mainly specific cytotoxic cells are involved. The generation of cytotoxic cells is performed *in vivo* by sensitisation of donor fish using allogeneic grafting. Primary antigen recognition, antigen processing, antigen presentation and proliferation of cytotoxic cells is already completed during sensitisation. Thus, this assay is a good tool to study the influence of temperature on specific cytotoxic cells.

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The graft-versus-host reaction (GVHR) is a representative phenomenon of *in vivo* cell-mediated immunity against alloantigens in which cytotoxic T cells play a major role. GVHR was first described in fish by Nakanishi and Ototake (1998) in a triploid ginbuna and tetraploid ginbuna-goldfish hybrid system. Like in CMC, donors of effector cells first have to be grafted to generate cytotoxic cells which in turn cause GVHR in the allogeneic target fish. In contrast to *in vitro* CMC, both sensitisation and the cytotoxic reaction is performed *in vivo*.

The present study was undertaken to assay the temperature related suppression of specific CMC *in vitro* and *in vivo*.

In vitro CMC assay was performed as previously described (Fischer *et al*., 1998*a*). Briefly, clonal triploid ginbuna crucian carp (S3n; *Carassius auratus langsdorfii*) originating from lake Suwa in Nagano Prefecture, Japan, were sensitised by grafts from tetraploid hybrids (S4n) obtained by insemination of S3n fish with goldfish (*Carassius auratus*) sperm (Nakanishi, 1987). Tetraploid fish are genetically tolerant of triploid cells but grafted triploid cytotoxic cells can react against allogeneic tetraploid cells. S3n fish were sensitised four times by S4n grafts, first by erythrocyte injection, second by erythrocyte injection 30 days after primary sensitisation, third by erythrocyte injection (5×10^6 cells per fish each) 14 days after secondary sensitisation and fourth by scale grafting (according to Nakanishi, 1987) 9 days after the third sensitisation. Water temperature was adjusted to 25° C during the sensitisation period. Sensitised S3n effector cells were obtained by density gradient centrifugation of blood using Percoll (Sigma; $\rho = 1{\cdot}085$ g/cm^3). 82% (S.D. ± 6%) of the isolated PBL were lymphocytes. A haemoglobin release assay was performed to measure the cytotoxicity of sensitised PBL against allogeneic erythrocytes (Fischer *et al*., 1998*a*). S3n effector cells were incubated with S4n erythrocytes at 15, 20 and 25° C for 5 h. Haemoglobin released from killed erythrocytes was detected using tetramethylbenzidine.

Results from haemoglobin release assay were compared statistically by an f-test followed by a two-tailed Student's *t*-test and were considered to be significantly different at $P \leq 0{\cdot}05$.

For GVHR experiments S3n effector cell donors were sensitised twice by scale grafting from S4n fish. S3n graft recipients were kept at 25° C. Scale grafts from S4n fish were rejected from S3n fish within 4–6 days. To induce GVHR, S4n fish were injected intraperitoneally with 2×10^6 PBL from sensitised S3n fish. S4n fish were kept at 15, 20 and 25° C. The result of GVHR was estimated comparing survival times after GVHR induction.

In vitro CMC of sensitised S3n effector PBL toward allogeneic S4n erythrocytes was dependent on the temperature at which the cells were cultured (Fig. 1). Particularly at ratios 1:20 and 1:40, killing of S4n erythrocytes by S3n PBL was more efficient at 25° C than at lower culturing temperatures.

Injection of sensitised effector PBL from S3n fish to S4n recipients resulted in graft-versus-host disease (GVHD). GVHD was characterised by paleness of gills, scale protrusion, ascites, haemorrhages and necrosis of the ventral part of skin, muscle layers and peritoneum. At autopsy fish showed peritonitis and necrosis of intestine and liver. Death of tetraploid fish occurred earliest when fish were cultured at 25° C; fish kept at 15° C exhibited a lower death rate and died later than those kept at higher temperatures (Table 1).

In vitro CMC against allogeneic erythrocytes as well as GVHR was inducible only if effector cell donors were previously sensitised by allogeneic grafting. Unsensitised effector cells do not cause *in vitro* CMC or *in vivo* GVHD (Nakanishi & Ototake, 1998; Fischer *et al*., 1998a). Thus, the need for sensitisation suggests that cells responsible for *in vivo* and *in vitro* CMC are specific cytotoxic cells rather than NCC.

Sensitisation of effector cell donors in which helper and/or accessory cells are indispensable for the generation of cytotoxic cells was carried out *in vivo* at constant temperature whereas cytotoxic effector cell function was assayed at different temperatures *in vitro* and *in vivo*. Therefore it can be suggested that mainly cytotoxic effector cells were suppressed at low temperature. For *in vitro* CMC it is unlikely that effector cells proliferate over the short time (5 h) of incubation with target cells, but cytokines

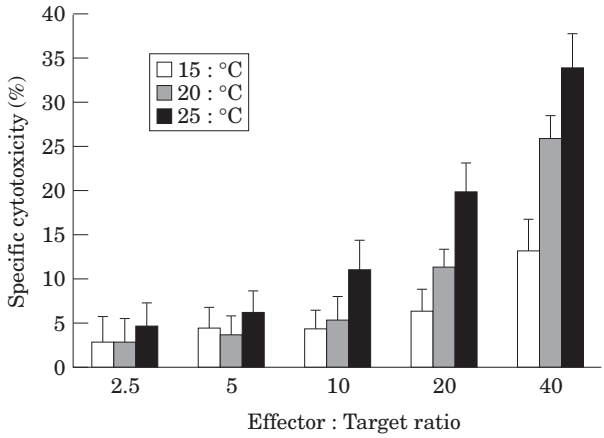

Fig. 1. Dependence of *in vitro* CMC on temperature. Pooled effector PBL were obtained from S3n fish (n=14). Vertical bars indicate standard deviation of triplicate wells. Values for specific cytotoxicity within ratios 1:20 and 1:40 are statistically different ($P \leq 0{\cdot}05$) between different culturing temperatures. Values for specific cytotoxicity within temperature groups of 20° C and 25° C are statistically different between ratios 1:10; 1:20 and 1:40 ($P \leq 0{\cdot}05$).

Table 1. Temperature dependence of GVHR

Day	Procedure	Survival/death at culturing temperature I: 25° C	II: 20° C	III: 15° C
− 21 d	First grafting			
− 7 d	Second grafting			
0 d	Effector cell injection			
18 d		fish 1 – †		
21 d		fish 2 – †		
24 d		fish 3 – †		
33 d			fish 1 – †	
38 d			fish 2 – †	
45 d			fish 3 – †	
52 d			fish 4 – †	
56 d				fish 1 – †
71 d	experiment finished	1 of 4 survived*	0 of 4 survived	3 of 4 survived**

*Fish I/4 showed severe clinical signs of GVHD from day 17 after induction of GVHR. At the end of the experiment this fish completely recovered and gained weight

**Surviving fish in this group showed no clinical signs at the end of the experiment

†Lethal outcome

released by helper and/or accessory cells could have stimulated cytotoxic cells. GVHR needs not hours but days to be observed. Thus, helper and accessory cells injected together with the specific cytotoxic cells may have influenced the latter and effector cells could even have proliferated in the recipient. This, however, needs further clarification since suppression of antibody formation in fish at low temperature can be attributed to a suppression of helper cells (Weiss & Avtalion, 1977; Miller *et al*., 1986; Bly & Chem, 1991; Vallejo *et al*., 1992). Separation of cytotoxic cells by specific sorting

using monoclonal antibodies against cytotoxic cells will help to solve this problem. Unfortunately, those antibodies are not yet available for fish.

A certain number of sensitised effector cells is required to induce GVHR and the death rate increases with the number of injected cells (Nakanishi & Ototake, 1998). In the present experiments one of four fish reared at 25° C showed severe clinical signs of GVHD but finally recovered, and fish kept at 15° C showed weak GVHD but only one of four fish died. The *in vivo* half life of lymphocytes from ginbuna crucian carp is less than 23 days (Fischer *et al*., 1998*b*). However, parallel experiments have shown, that S3n PBL can proliferate in S4n recipients to a certain extent (unpublished data). If target fish resist the attacks of injected effector cells over a certain period during which effector cell number decreases substantially, they will ultimately survive GVHD.

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