# コイ白血球を用いたin vitro抗体産生応答


| 誌名 | 養殖研究所研究報告 = Bulletin of National Research Institute of Aquaculture |
| --- | --- |
| ISSN | 03895858 |
| 著者 | Moreira, P.B.<br>中西, 照幸 |
| 巻/号 | 25号 |
| 掲載ページ | p. 43-53 |
| 発行年月 | 1996年3月 |



農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

AgriKnowledge

Bull. Natl. Res. Inst. Aquacult. No. 25, 43-53 (1996)

# *In Vitro* Humoral Immune Response of Carp *Cyprinus carpio* L. Leucocytes

Paloma B. Moreira*[1)] and Teruyuki Nakanishi*[2)]

(Received January 10, 1996)

An *in vitro* culture system was developed to study humoral immune response of carp kidney cells, and using this system *in vitro* plaque-forming cell (PFC) response was examined at incubation temperatures of 10, 15, 25 and 30°C. Both head and trunk-kidney cells showed similar kinetics and magnitude of *in vitro* generation of PFC. Both the primary and secondary PFC responses specific to horse red blood cell (HRBC) were observed. The peak in the secondary response was three times higher than in the primary response at all temperatures examined. At 15°C PFC generation was quicker in the secondary (at day 6) than in the primary response (at day 9), although at both 25 and 30°C PFC appeared after the same latent period (3 days) in both the primary and secondary responses. The higher the temperature, the shorter the period before the peak response was reached: three days at 30°C, six days at 25°C and twelve days at 15°C. At 10°C PFCs did not appear until 18 days in culture.

*Key words*: antibody-producing cell, carp, *Cyprinus carpio*, *in vitro* antibody production, kidney cell, plaque-forming cell, temperature

## Introduction

The high degree of homology of fish immune system with that of higher vertebrates has received further confirmation through *in vitro* study. Functional *in vitro* system allows the manipulation of immunocompetent cells in ways not possible *in vivo*. Thus, using *in vitro* system well-known characters of the mammalian immune system have also been demonstrated in fish: mitogen response (Etlinger *et al.*, 1976; Caspi *et al.*, 1984-a; Clem *et al.*, 1984; Tillit *et al.*, 1988), mixed leukocyte response (MLR, Etlinger *et al.*, 1977; Caspi and Avtalion, 1984-b; Miller *et al.*, 1986; Kaastrup *et al.*, 1988), collaboration of Ig+ with Ig− cell populations (Sizemore *et al.*, 1984; Miller *et al.*, 1985), primary and secondary *in vitro* humoral responses (Miller and Clem, 1984-a, 1984-b; Anderson *et al.*, 1986), production of interferon-like molecules (Graham and Secombes, 1990) and other cytokines (Grondel and Harmsen, 1984; Caspi and Avtalion, 1984-c).

A number of *in vivo* studies have shown that both humoral and cell-mediated immune responses in fishes are temperature dependent (Avtalion *et al.*, 1976; Kikuchi *et al.*, 1983; Nakanishi, 1983, 1985). Collateral factors, such as increased output of corticosteroids at low temperatures rather than a direct temperature-dependent effect on lymphocyte, have been suggested to influence immune response (Ellis,

*[1)] Experimental Centre Vilaxoan, Apdo. 208, Vilagarcia de Arousa, Pontevedra, Spain (パロマ B. モレイーラ)
*[2)] National Research Institute of Aquaculture, Tamaki, Mie 519-04 Japan (中西照幸：養殖研究所玉城庁舎)

1981). *In vitro* studies make it possible to study the effect of temperature on immune response excluding the collateral factors. The effect of temperature on *in vivo* humoral response of carp has been extensively studied (Avtalion, 1969; Avtalion *et al.*, 1973; Rijkers *et al.*, 1980-a, 1980-c). However, no *in vitro* study on humoral response has been made in carp, though several studies have been reported in catfish (Miller and Clem, 1984-b) and rainbow trout (Anderson *et al.*, 1986; Kaattari *et al.*, 1986). In the present study we describe the *in vitro* generation of plaque-forming cell (PFC) response in carp and the kinetics of primary and secondary *in vitro* immune responses, together with the effect of temperature upon it.

## Materials and Methods

### *Animals*

Carp (*Cyprinus carpio*), weighing 100 to 500 g, were obtained from a commercial fish farm and held in outdoor ponds with spring water in National Research Institute of Aquaculture. They were transferred to indoor tanks with running water at $25 \pm 0.5$°C and acclimatized for at least two weeks before being used in experiments. Commercial pelleted food was given daily.

### *Cell Suspensions*

Carp were anaesthetized with ethyl p-amino benzoate (100 mg/l) prior to manipulation, and bled as much as possible via the caudal blood vessels to avoid the contamination of organs with blood. Tissues were removed and rinsed with the washing medium in small plastic Petri dishes on ice. The washing medium was prepared according to Liewes *et al.* (1982). Briefly, 128 ml of deionized water was added to 1 l of RPMI-1640 (Flow Labo. Inc., USA) to adjust to carp osmolarity (242 mOsm). Then pH was adjusted to 7.4 with $NaHCO_3$, and L-glutamine and 10 IU/ml of heparin were added to the medium containing penicillin (100 IU/ml) and streptomycine (100 $\mu$g/ml). The preparation of cell suspensions was made by teasing tissues and sieving them through the stainless steel mesh (87.5 $\mu$m) to remove coarse aggregates. Then cells were washed twice by centrifugation at $300 \times g$ for 10 min at 4°C, counted and adjusted to the appropriate density. Cell viability was greater than 95% as assessed by the trypan blue exclusion test.

### *In vitro culture*

Cells were cultured in flat bottomed 24-well tissue culture plates or round bottomed 96-well microculture plates (Corning) at the seeding density of $10^6$ cells/ml in 1 ml or 200 $\mu$l of culture medium/well, respectively. Tissue culture medium (TCM) was prepared adding 10% pooled carp serum (PCS) or a mixture of 5% PCS and 2% fetal bovine serum (FBS) to the washing medium. Carp serum was heat inactivated (20 min at 45°C) and filtrated through 0.45 $\mu$m Millipore filters for decontamination. The plates were incubated at appropriate temperatures for at most 18 days in a humidified 5% $CO_2$- 95% air atmosphere. Pooled cells from head and trunk-kidney (50% from each of them) were used except the experiment shown in Fig. 1.

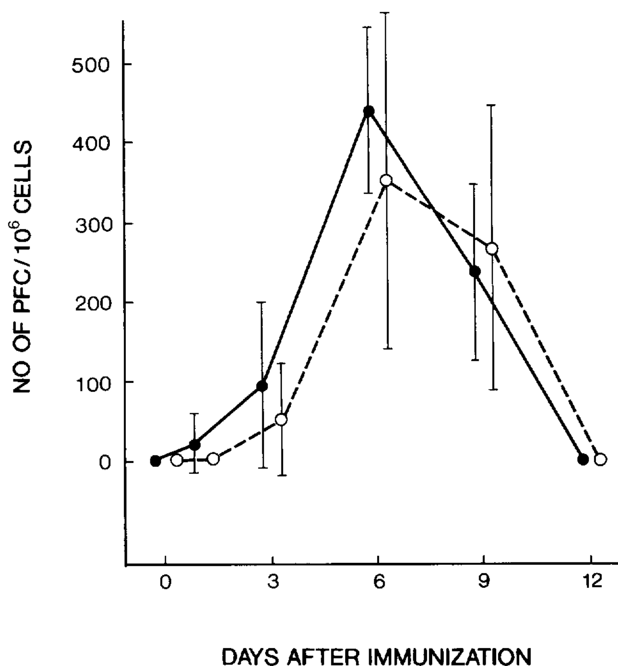


**Fig. 1.** Kinetics of the primary anti-HRBC PFC responses of head (●——●) and trunk (○······○) kidney cells. Cells were cultured at 25°C. Data are expressed as the mean number of *in vitro* generated PFC/$10^6$ lymphoid cells. Ratio of cells to HRBC was 1:4. Vertical bars indicate standard deviation of three fish.

*Immunization*

Horse red blood cells (HRBC) in Alsever's solution (Teikoku Zoki Ltd., Tokyo) were washed twice with phosphate-buffered saline (PBS) and adjusted to 20% suspension. Fish used as sources of cells for secondary *in vitro* response were given three i.p. injections of 20% HRBC at two-day intervals with 5 $\mu$l/g body weight. They were used when the serum titer had decreased (at least six weeks after the first injection).

For *in vitro* immunization HRBC in TCM were delivered into wells of microculture plates at different ratios of lymphoid cells to HRBC. Optimal cell-antigen ratio for PFC response was examined using cell-HRBC ratio of 1/1, 1/2, 1/4 and 1/8.

To check the specificity of the response, bovine serum albumin (BSA, Sigma, Fraction IV) was used. BSA dissolved in TCM was filtrated before delivering into wells. In a preliminary experiment the optimal BSA concentration was determined using BSA concentrations of 0.3, 3, 30 and 100 $\mu$g/ml. Thus, BSA concentration of 3 to 30 $\mu$g/ml was found to be the optimal dose. BSA dose of 100 $\mu$g/ml was toxic to cells and no PFC response was obtained at the dose of 0.3 $\mu$g/ml. Cells were routinely cultured with two doses of antigen and the data were expressed as the mean PFC/$10^6$ lymphoid cells at the antigen dose yielding the maximal response.

*Temperature*

*In vivo* immunization was made at 25°C. *In vitro* trials were undertaken at the following temperatures: 10, 15, 25 and 30°C for the primary immunization and 15, 25 and 30°C for the secondary immunization.

*Plaque-forming cell (PFC) assay*

PFC assay was performed in liquid according to Cunningham and Szenberg (1968) with minor modifications, as reported for rockfish (Nakanishi, 1986). In short, for the direct haemolytic PFC assay a mixture of HRBC, lymphoid cells and absorbed carp plasma as a complement (C') source were pipetted into parafilm chambers (three chambers/slide). Erythrocytes and lymphoid cells were resuspended in TCM without serum, since inactivated carp serum added to the mixture including homologous C' can inhibit plaque formation (Rijkers *et al.*, 1980-b). The slides were incubated at 25°C for two hours and then plaques were scored under a phase-contrast microscope. The controls were as follows: slides with cell free medium, antigen and C' or slides with cells and antigen in the absence of C'. Triplicate cultures on a slide were assayed and the data expressed as the mean number of *in vitro* generated plaques per $10^6$ lymphoid cells.

Carp plasma, absorbed with equal volume of packed HRBC for one hour at 0°C, was used as C' source. When necessary, it was kept at −80°C. Every batch of plasma, newly prepared or thawed was tested for natural haemolytic activity prior to use and for the PFC assay a dilution higher than that yielding natural lysis was chosen. Final concentration of the C' was usually 5%, varying between 2.5 and 10%.

For the indirect PFC assay HRBC were coupled to BSA with 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide HCl (ECDI, Sigma Chemical) by the method of Golub *et al.* (1968) modified by Kipp and Miller (1980).

*Analysis of data*

The statistical treatment of the results was carried out using the SPSS/PC+ V2.0 (SPSS Inc.) package.

## Results

*Comparison of primary response between trunk and head kidney cells*

Fig. 1 shows the primary *in vitro* anti-HRBC responses of trunk and head kidney cells at 25°C. No significant difference was found between the two tissues with regard to kinetics and magnitude (paired-samples t-test: $P<0.05$). The peak of the response occurred after six days in culture, and no plaques were detected after twelve days. For both groups, every mean value of the activity had a large standard deviation reflecting high variation of the response among individuals.

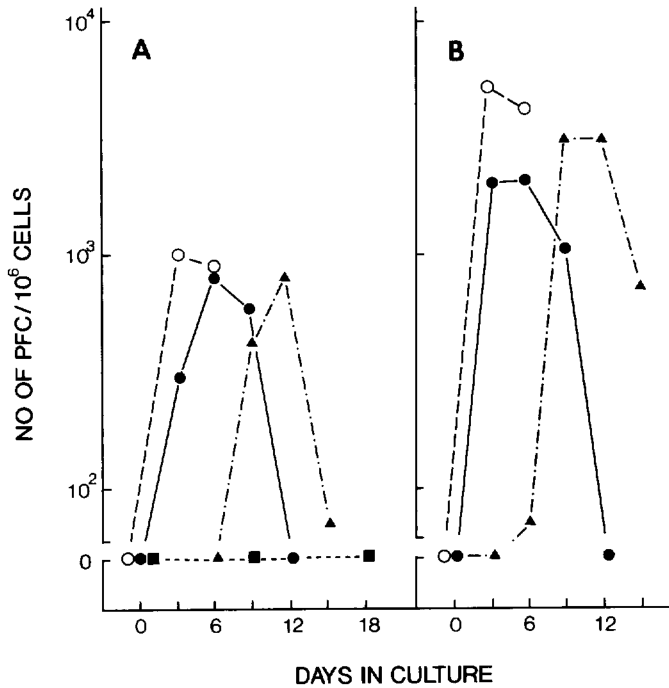


**Fig. 2.** Comparison of the primary (A) and secondary (B) anti-HRBC PFC response in carp kidney cells and the effects of incubation temperature on kinetics of the response. Data are expressed as the mean number of PFC/$10^6$ lymphoid cells in semilogarithmic scale. Ratio of cells to HRBC was 1:4. Each point represents the mean response of three fish. 10°C, ■; 15°C, ▲; 25°C, ●; 30°C, ○.

*Primary and secondary responses in pooled kidney cells and effect of temperature*

Fig. 2 shows the primary (Fig. 2-A) and secondary (Fig. 2-B) *in vitro* anti-HRBC responses at four different temperatures of 10, 15, 25 and 30°C. The peak in the secondary response was three times higher than in the primary response at all temperatures examined (independent-samples t-test: $P<0.01$), although the magnitude of the peak response did not significantly differ among three temperatures in both primary and secondary responses. At 15°C the first plaques were detected earlier in the secondary (at day 6) than in the primary response (at day 9), although at both 25 and 30°C it appeared on the same day (at day 3) for the primary and secondary responses. Fig. 3 summarizes the relationship between *in vitro* temperature and the day of the peak PFC response to HRBC and shows a linear relationship ($r=-1$) between them. The higher the temperature, the shorter the time before the peak PFC response was reached, and the periods required for the peak responses to HRBC were about 3, 6 and 12 days at 30, 25 and 15°C, respectively. At 10°C plaques did not appear during the experimental period (18 days of culture).

*Effect of antigen-dose on in vitro PFC response*

*In vitro* PFC response to HRBC was dependent on antigen dose, though optimal dose varied among

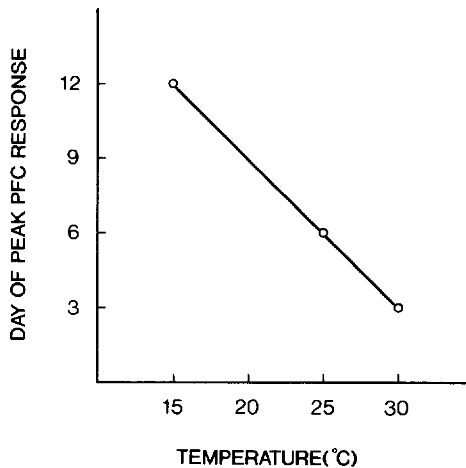


**Fig. 3.** Relationship between *in vitro* temperatures and the day of the peak PFC response to HRBC between 15 and 30°C. Regression line: $y = -1.667x + 35.0$. The linear regression coefficient (r) was $-1.0$.

**Table 1.** Effect of antigen dose on primary *in vitro* PFC response of carp kidney cells to HRBC.

| Fish No. | Control* | Ratio of cells to HRBC | | | |
|---|---|---|---|---|---|
| | | 1/1 | 1/2 | 1/4 | 1/8 |
| 1 | 20.8±16.2 | 83.3±13.3 | 146.2±16.8 | 688.0±14.5 | 229.3± 8.6 |
| 2 | 21.0±13.3 | 41.7±13.8 | 250.0± 0.0 | 833.3±18.4 | 333.3±27.5 |
| 3 | 0.0± 0.0 | 125.0±23.8 | 417.0±13.5 | 250.0±23.8 | 83.3± 7.6 |
| 4 | 20.8±16.2 | 20.8±16.2 | 250.3±61.3 | 939.3±35.3 | ND |

Cells were cultured at 30°C with HRBC and assayed on day 3. * Cells were cultured without antigens. Data are expressed as mean±SD of PFC/$10^6$ cells. n=3 (number of chambers). ND=Not done.

trials. As shown in Table 1 cell-HRBC ratio of 1/2 and 1/4 yielded optimal responses, while 1/1 and 1/8 always gave suboptimal response.

*Specificity of in vitro PFC response*

To test the specificity of the primary immune response cells immunized with BSA or HRBC and non-immunized control cells were assayed by using HRBC or BSA coupled to HRBC as indicator systems. As shown in Table 2 significantly larger amount of plaques occurred in immunized cells tested with the same antigen indicator system than in immunized cells tested with different antigen indicator system and non-immunized samples (one way ANOVA and Tukey-HSD multiple range test, $P<0.05$). The primary *in vitro* response was higher and more specific for BSA than for HRBC.

Low, but significant number of PFC were observed in half of the individuals of which cells were cultured *in vitro* without antigen (Table 3). Such background PFC was already detected in the leukocyte suspensions prior to culture and kept constant during the incubation period (Table 3). To assess the possibility of the plaque generation caused by the serum supplementation we added various combinations

**Table 2.** Specificity of the primary *in vitro* PFC response of carp kidney cells.

| Fish No. | Antigen* | Indicator system | |
|---|---|---|---|
| | | HRBC | BSA-HRBC |
| 1 | HRBC | 729.3±33.1 | 166.7± 14.0 |
| | BSA | 125.0± 0.0 | 875.3± 32.0 |
| | None | 333.7± 6.9 | 92.0± 30.6 |
| 2 | HRBC | 593.0± 9.9 | 104.2± 8.5 |
| | BSA | 129.2±36.9 | 1312.7±102.7 |
| | None | 108.7± 8.4 | 41.7± 8.5 |
| 3 | HRBC | 438.0±12.1 | 105.0± 24.2 |
| | BSA | 82.5± 0.0 | 708.7± 76.5 |
| | None | 125.2±12.1 | 20.8± 16.2 |

Cells were cultured at 25°C and assayed on day 6. Ratio of cells to HRBC was 1:4. * Antigen dose of BSA used were as follows: 3 μg/ml for fish 1 and 2, and 30 μg/ml for fish 3. Data are expressed as mean±SD of PFC/$10^6$ cells (n=3).

**Table 3.** Background level of anti-HRBC plaques in carp kidney cells.

| Culture Period (Days) | 0 | 6 | 6 |
|---|---|---|---|
| Fish No. | Non-immunized | | Immunized* |
| 1 | 125.0± 0.0 | 108.7± 9.4 | 593.0± 9.9 |
| 2 | 0.0 | 0.0 | 452.3±15.8 |
| 3 | 41.7±16.8 | 41.7±16.8 | 1106.3±69.5 |
| 4 | 0.0 | 0.0 | 417.0±16.7 |

Cells were cultured at 25°C. * The ratio of cells to HRBC was 1:4. Data are expressed as mean±SD of PFC/$10^6$ cells (n=3).

of serum sources such as 10% FBS, 5% FBS-2% PCS, 2% FBS-5% PCS and 10% PCS to the culture system. However, we could not find any significant plaque generation due to these serum supplementation (data not shown).

## Discussion

The present study confirms the known importance of carp kidney in antibody production *in vivo* (Rijkers *et al.*, 1980-a, 1980-b) and proves that both head and trunk-kidney are fully immunocompetent tissues. The functional similarity between both organs found in this work is in agreement with the study by Liewes *et al.* (1982) showing the same pattern of *in vitro* mitogenic responses in carp trunk and head-kidney cells.

Clear primary and secondary responses specific to HRBC were observed at incubation temperatures

of 15, 25 and 30°C. Background plaques which appeared in cells prior to culture didn't show any changes during incubation. Though non-specific stimulation has been reported by serum supplement in both fish (Kaattari and Yui, 1987) and mammalian (Coutinho *et al.*, 1973) culture systems, similar levels of plaques were found even in serum-free controls in our study. Therefore, these plaques were considered not to be generated *in vitro*, but to be naturally occurring PFC caused by unknown environmental antigen sharing some antigenic determinant with HRBC. "Natural" antibodies to erythrocyte antigen have been reported to occur in fish (Chiller *et al.*, 1969; Avtalion *et al.*, 1973; Miller and Clem, 1984-a).

Criteria to asses immunological memory in vertebrates are higher peak titer of antibody and shorter latent period in the secondary immune response. The first criteria is fulfilled with the response at all three assayed temperatures. The second is apparent at 15°C but not at 30°C, where the response was so quick that the exact time of peak was not determined in this study. The existence of the anamnestic character in the response at 15°C in the present study is not in agreement with the study by Rijkers *et al.* (1980-a) who found that anamnestic response was lost under 18°C in carp immunized *in vivo* with SRBC. They attributed this loss due to some effect of temperature on the formation of memory cells. We must notice that they kept carps at 18°C throughout the experiment, while we undertook *in vivo* primary immunization at 25°C. A number of studies in carp (see Avtalion, 1969) have shown the normal secondary response at low temperatures (10–14°C) as far as fish had been maintained at higher temperatures (20–25°C) during the first antigenic stimulation.

Different workers employing a variety of antigens have reported a complete suppression of antibody formation at lower temperatures both *in vivo* in carp (see Avtalion *et al.*, 1973, 1976) and *in vitro* in catfish (Miller and Clem, 1984-b); while other reports have indicated only a delay in time of appearance of antibody or PFC without suppression *in vivo* in carp (Rijkers *et al.*, 1980-a, 1980-c) and flounder (Stolen *et al.*, 1984). We found delays in the peak response for both *in vitro* primary and secondary responses at low temperatures and could not detect any PFCs at 10°C as long as 18 days of culture. Rijkers *et al.* (1980-c) reported that humoral anti-SRBC response occurred *in vivo* at temperatures between 8 and 28°C, and the lower the temperature the later was observed the peak response, though not decreasing in magnitude. According to the regression line calculated from the present data (Fig. 3) the response should attain to the peak by day 18 even when cultured at 10°C. However, if the slope of the regression line *in vitro* became greater at temperatures lower than 18°C as found *in vivo* by Rijkers *et al.* (1980-a), the peak might be reached later. In the present study cultures were done until day 18, but not further due to the lower viability of cells. Hence, whether PFC response to HRBC *in vitro* is completely suppressed at lower temperature or not still remains to be clarified.

The antigen-dose dependence of the response found in this study has also been reported for catfish in *in vitro* immunization experiments (Miller *et al.*, 1984-a). Differences in the antibody response are known to occur *in vivo* according to antigen dosage, route of administration and timing of booster injections (see Ellis, 1981, Nakanishi, 1982). Avtalion *et al.* (1980) even found immune suppression in carp primed with the high doses of BSA antigen at low temperature.

## Acknowledgments

We are grateful to Dr. Y. Inui for reviewing the manuscript. We would like to thank Dr. T. Hara for his encouragement. This work was supported in part by Xunta de Galicia (Spain), Grant-in Aid (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries (BMP 96-V-2-2-4) and the Rotary Club, from Ise-shi (Japan).

## References

Anderson, D. P., O. W. Dixon and E. F. Lizzio 1986. Immunization and culture of rainbow trout organ sections *in vitro*. Vet. Immunol. Immunopathol. **12**: 203–211.

Avtalion, R. R. 1969. Temperature effect on antibody production and immunological memory in carp (*Cyprinus carpio*) immunized against bovine serum albumin (BSA). Immunology **17**: 927–931.

Avtalion, R. R., A. Wojdani, Z. Malik, R. Shahrabani and M. Duczyminer 1973. Influence of environmental temperature on the immune response of fish. Curr. Top. Microbiol. Immunol. **61**: 1–35.

Avtalion, R. R., E. Weiss and T. Moalem 1976. Regulatory effect of temperature upon immunity in ectothermic vertebrates. pp. 227–238, In "Comparative Immunology", ed. by J. H. Marchalonis, Blackwell Scientific Publications, Oxford.

Avtalion, R. R., A. Wishkovsky and D. Katz 1980. Regulatory effect of temperature on specific suppression and enhancement of the humoral response in fish. pp. 113–121, In "Phylogeny of Immunological Memory", ed. by M. J. Manning, Elsevier/North-Holland Biomedical Press, Amsterdam.

Caspi, R. R., R. Shahrabani, T. Kehati-Dan and R. R. Avtalion 1984-a. Heterogeneity of mitogen-responsive lymphocytes in carp (*Cyprinus carpio*). Dev. Comp. Immunol. **8**: 61–70.

Caspi, R. R. and R. R. Avtalion 1984-b. The mixed leukocyte reaction (MLR) in carp: Bidirectional and unidirectional MLR responses. Dev. Comp. Immunol. **8**: 631–637.

Caspi, R. R. and R. R. Avtalion 1984-c. Evidence for the existence of an IL-2 like lymphocyte growth promoting factor in a bony fish, *Cyprinus carpio*. Dev. Comp. Immunol. **8**: 51–60.

Chiller, J. M., H. O. Hodgins, and R. S. Weiser 1969. Antibody response in rainbow trout (*Salmo gairdneri*). II. Studies on the kinetics of antibody-producing cells and on complement and natural hemolysin. J. Immunol. **102**: 1202–1207.

Clem, L. W., E. Faulmann, N. W. Miller, C. Ellsaesser, C. J. Lobb and M. A. Cuchens 1984. Temperature mediated processes in teleost immunity: Differential effects of *in vitro* and *in vivo* temperatures on mitogenic responses of channel catfish lymphocytes. Dev. Comp. Immunol. **8**: 313–322.

Coutinho, A., G. Moller, J. Anderson and W. W. Bullock 1973. *In vitro* activation of mouse lymphocytes in serum free medium: effect of T and B cell mitogens on proliferation and antibody synthesis. Eur. J. Immunol. **3**: 299.

Cunningham, A. J. and A. Szenberg 1968. Further improvements in the plaque technique for detecting single antibody-producing cells. Immunology. **14**: 599–600.

Ellis, A. E. 1981. Stress and the modulation of the defense mechanisms in fish. pp. 147–169, In "Stress and Fish", ed. by A. D. Pickering, Academic Press, Oxford.

Etlinger, H. M., H. O. Hodgins, and J. M. Chiller 1976. Evolution of the lymphoid system. I. Evidence for lymphocyte heterogeneity in rainbow trout revealed by organ distribution of mitogenic responses. J. Immunol. **116**: 1547–1553.

Etlinger, H. M., H. O. Hodgins, and J. M. Chiller 1977. Evolution of the lymphoid system. II. Evidence for immunoglobulin determinants on all rainbow trout lymphocytes and demonstration of mixed leukocyte reaction. Eur. J. Immunol. **17**: 881–887.

Golub, E. S., R. I. Mishell, W. O. Weigle and R. W. Dutton 1968. A modification of the hemolytic plaque assay for use with protein antigens. J. Immunol. **100**: 133.

Graham, S. and C. J. Secombes 1990. Do fish lymphocytes secrete interferon-$\gamma$?. J. Fish Biol. **36**: 563–574.

Grondel, J. L. and E. G. M. Harmsen 1984. Phylogeny of interleukins: growth factors produced by leucocytes of the cyprinid fish, *Cyprinus carpio* L. Immunology 52: 477–482.

Kaastrup, P., B. Nielsen, V. Horlyck, and M. Simonsen 1988. Mixed lymphocyte reactions (MLR) in rainbow trout (*Salmo gairdneri*) sibling. Dev. Comp. Immunol. 12: 801–808.

Kaattari, S. L., M. J. Irwin, M. A. Yui, R. A. Tripp, and J. S. Parkins 1986. Primary *in vitro* stimulation of antibody production by rainbow trout lymphocytes. Vet. Immunol. Immunopathol. 12: 29–38.

Kaattari, S. L. and M. A. Yui 1987. Polyclonal activation of salmonid B lymphocytes. Dev. Comp. Immunol. 11: 155–165.

Kikuchi, S., A. Imazumi and N. Egami 1983. The effect of temperature on the immunological memory against allograft transplantation in the fish *Oryzias latipes*. J. Fac. Sci. Imp. Univ. Tokyo. 15: 325–328.

Kipp, D. and A. Miller 1980. Preparation of protein-conjugated red blood cells with ECDI (modification). pp. 103–105, In "Selected Methods in Cellular Immunology", eds. by Barbara Mishell & Stanley M. Shiigi, W. H. Freeman and Company, New York.

Liewes, E. W., R. H. Vam Dam, M. G. Vos-Maas, and R. Bootsma 1982. Optimization of *in vitro* stimulation of carp *Cyprinus carpio* L. leukocytes. Vet. Immunol. Immunopathol. 3: 325–343.

Miller, N. W. and L. W. Clem 1984-a. Microsystem for *in vitro* primary and secondary immunization of channel catfish *Ictalurus punctatus* leukocytes with hapten-carrier conjugates. J. Immunol. Meth. 72: 367–379.

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

Miller, N. W., R. C. Sizemore, and L. W. Clem 1985. Phylogeny of lymphocyte heterogeneity: the cellular requirements for *in vitro* antibody responses of channel catfish leukocytes. J. Immunol. 134: 2884–2888.

Miller, N. W., A. Deuter, and L. W. Clem 1986. Phylogeny of lymphocyte heterogeneity: The cellular requirements for the mixed leucocyte reaction with channel catfish. Immunology. 59: 123–128.

Nakanishi, T. 1982. The immune response of the rock fish, *Sebasticus marmoratus*-I. Response of antibodies and hemolysin plaque-forming cells to SRBC. Bull. Natl. Res. Inst. Aquaculture 3: 81–89.

Nakanishi, T. 1983. The immune response of the rock fish, *Sebasticus marmoratus*-II. Effects of temperature on the antibody production and plaque-forming cell response to SRBC. Bull. Natl. Res. Inst. Aquaculture 4: 121–129.

Nakanishi, T. 1985. The immune response of the rock fish, *Sebasticus marmoratus*-III. Eye transplantation immunity and effects of temperature on scale allograft rejection. Bull. Natl. Res. Inst. Aquaculture 8: 43–50.

Nakanishi, T. 1986. Antibody producing cells in the marine teleost, *Sebasticus marmoratus*. Organ distribution and morphology. pp. 333–342, In: "Pathologie en Aquaculture Marine", eds. by C. P. Vivares, J. R. Bonami and E. Jaspers, European Aquaculture Society, Special Publication No. 9. Bredene, Belgium.

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

Rijkers, G. T., E. M. H. Frederix-Wolters, and W. B. van Muiswinkel 1980-b. The haemolytic plaque assay in carp (*Cyprinus carpio*). J. Immunol. Meth. 33: 79–86.

Rijkers, G. T., J. A. M. Wiegerinck, R. van Oosterom and W. B. van Muiswinkel 1980-c. Temperature dependence of humoral immunity in carp (*Cyprinus carpio*). Proceedings of the 1st Congress of Developmental and Comparative Immunology, Aberdeen. (Ed. by J. B. Solomon). pp. 477–482, Pergamon Press. Oxford.

Sizemore, R. C., N. W. Miller, M. A. Cuchens, C. J. Lobb, and L. W. Clem 1984. Phylogeny of lymphocyte heterogeneity: the cellular requirements for *in vitro* mitogenic responses of channel catfish leukocytes. J. Immunol. 133: 2920–2924.

Stolen, J. S., T. Gahn, V. Kasper, and J. J. Nagle 1984. The effect of environmental temperature on the immune response of a marine teleost (*Paralichthys dentatus*). Dev. Comp. Immunol. 8: 89–98.

Tillit, D. E., J. P. Giesy and P. O. Fromm 1988. *In vitro* mitogenesis of peripheral blood lymphocytes from rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 89A: 25.

# コイ白血球を用いた *in vitro* 抗体産生応答

パロマ B. モレイーラ・中西　照幸

コイを用いて *in vitro* 抗体産生応答の手法を確立するとともに，1，2次応答及び温度の影響について検討した。抗原として馬赤血球と牛血清アルブミンを用いた。抗体産生細胞の検出は直接あるいは間接プラーク形成法により行った。頭腎細胞，体腎細胞いずれを用いた場合でも，抗体産生応答が認められた。25°C における1次応答においては，抗体産生細胞は3日目に出現し，6日目にピークに達成し，15日目には殆ど認められなくなった。この応答は著しく温度の影響を受け，ピークに達する時期は 15°C では12日目，30°C では3日目であった。また，10°C では実験期間中抗体産生細胞は認められなかった。2次応答において典型的な既往反応が観察され，1次応答に比べピークに達するまでの期間が短く，かつピーク時の抗体産生細胞数も数倍多く認められた。温度の影響は2次応答においても1次応答と同様に認められた。