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The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*)

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Summary. After immunization of carp with sheep red blood cells, the spleen accounts for only 5% of the total number of plaque-forming cells (PFC). In addition, thymus, peripheral blood and heart contained low numbers of PFC (<0·5, 1 and 0·5%, respectively). Pronephros and mesonephros were the major antibody-forming organs (53 and 40% of total PFC, respectively). The temperature dependence of the antibody-forming cell response in spleen, pronephros and mesonephros was studied in animals kept at 12–24°. Lowering temperatures induced a delay in the peak of the primary response but had no effect on the magnitude of the response. The temperature–peak day relationship indicated that there are steps in the primary immune response of carp differing in temperature sensitivity. The anamnestic character of the secondary response was clearly demonstrated at 24 and 20° but lost at 18°.

INTRODUCTION

Both cellular and humoral immune responses in poikilothermic vertebrates are temperature dependent. At low temperatures, allograft rejection proceeds at a relative slow rate (Hildemann, 1957; Cohen, 1966; Borysenko, 1970) and antibody production is diminished (Pliszka, 1939; Ambrosius & Schäker, 1964; Harris, 1973; Paterson & Fryer, 1974) or completely absent (Nybelin, 1935; Barrow, 1955; Avtalion, 1969a, b; Avtalion, Malik, Lefler & Katz, 1970). Upper and lower temperature limits of the immune response are closely related to the ecological temperature range of the species considered (Ridgway, Hodgins & Klontz, 1966; Tait, 1969; O'Neill, 1980). The relationship between temperature and allograft survival time in goldfish indicated that there were at least two temperature-sensitive stages in the process of allograft rejection (Hildemann, 1957). According to studies in different animals, the first phase of the immune response including antigen processing and recognition is relatively temperature-independent (Cone & Marchalonis, 1972; Avtalion, Wojdani, Malik, Shahrabani & Duczyminer, 1973). The first temperature-sensitive event might be the subsequent interaction between T- and B-like cells (Avtalion, Weiss & Moalem, 1976; Marchalonis, 1977; Cuchens & Clem, 1977). A possible explanation is a block in 'T-helper' activity (Cuchens, McLean & Clem, 1976) or an increase in 'T-suppressor' activity at low temperatures (Avtalion, Wishkovsky & Katz, 1980). The following cellular multiplication and differentiation of B-like cells are considered to be temperature-independent (Avtalion *et al.*, 1973). According to the same authors the second temperature-sensitive event is the synthesis and release of antibodies by plasma cells (Avtalion *et al.*, 1973).

Most studies mentioned above are centred around

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the influence of temperature on the effector phase of the immune response, e.g. antibody levels in serum or allograft survival time. In this paper, the kinetics of the plaque-forming cell response at different temperatures was studied in order to obtain more insight in the temperature dependence of the inductive and proliferative phase of the humoral immune response in poikilotherms.

MATERIALS AND METHODS

Animals

Outbred carp (*Cyprinus carpio*) were obtained from the Organization for Improvement of Inland Fisheries (O.V.B., Nieuwegein, The Netherlands) or bred in our laboratory. Animals from 6 to 18 months old, weighing 75–300 g, were used. They were kept in aquaria with aerated water (pH 7·4–7·8, $NO_2 < 0·3$ p.p.m., 4°DH) and fed daily on pelleted dry food (K30, Trouw & Co, Putten, The Netherlands). Animals were kept at 12, 16, 18, 20 or 24°. They were acclimatized for at least 2 weeks at each temperature before antigen injection.

Antigen and immunization

Sheep red blood cells (SRBC) were obtained from six animals. Pooled blood was mixed with an equal volume of Alsever's solution. The cells were washed three times with phosphate-buffered saline (PBS, pH 7·2) before use. Carp were immunized by an intramuscular (i.m.) injection of 10^9 SRBC in 0·05 ml PBS in the dorsal region.

Plaque-forming cell assay

Plaque-forming cells (PFC) were detected using the method described previously (Rijkers, Frederix-Wolters & van Muiswinkel, 1980a). Bream (*Abramis brama*) serum was used as complement source. Plaques were developed at 25° for 2 h unless stated otherwise. Viability of lymphoid cells was determined with the dye exclusion assay (0·2% trypan blue in PBS).

RESULTS

Organ distribution of PFC

In order to obtain an idea about the relative importance of different lymphoid organs of carp, the number of PFC in spleen, pronephros, mesonephros, heart, peripheral blood, thymus, liver and intestine was determined. Pronephros and mesonephros account for about 90% of the total PFC number after i.m. injection with SRBC (Table 1). The spleen accounts only for about 5% while thymus, heart and peripheral blood contain very low percentages of PFC ($\leq 1\%$). PFC were not detected in liver and intestine. Similar results were obtained when carp were injected intravenously with the same antigen. After oral administration of SRBC, considerable numbers of PFC were detected in pronephros and mesonephros and to a lesser degree in spleen but not in intestine.

PFC kinetics

The number of antibody-forming cells in suspensions of spleen, pronephros and mesonephros was determined at successive days after i.m. injection with 10^9

Table 1. Organ distribution of plaque-forming cells (PFC) in carp at 20°. Animals were injected twice with 10^9 SRBC (i.m.) with an interval of 1 month. The number of PFC was determined 12 days after the last injection (peak of the response)

Organ	PFC/10^6 white cells	PFC/organ	% of total PFC
Thymus	0·3 ± 0·04*	120	< 0·5
Spleen	25 ± 1	17,100	5
Pronephros	548 ± 30	184,000	53
Mesonephros	87 ± 4	138,000	40
Heart	71 ± 7	1,950	0·5
Peripheral blood	15 ± 1	3,000	1
Liver	0	0	—
Intestine	0	0	—

* Arithmetic mean ± SE ($n = 4$).

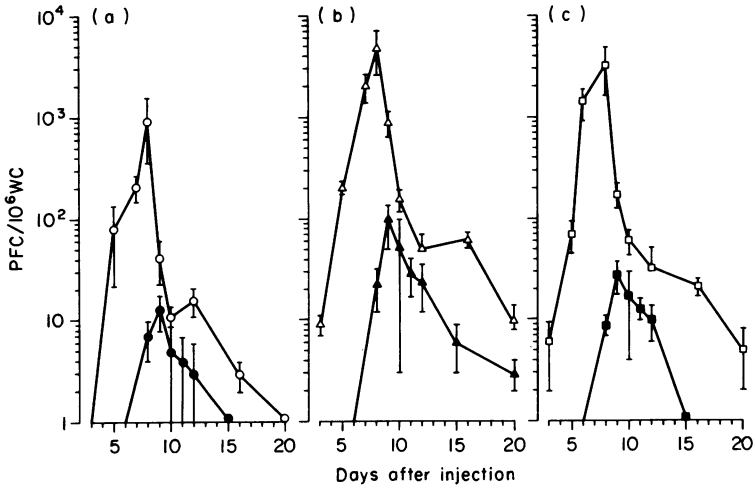

Figure 1. Kinetics of primary (filled symbols) and secondary (open symbols) anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 24°. Animals were injected twice with 10^9 SRBC. The interval between two i.m. injections was 1 month. PFC/10^6 WC = plaque-forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 SE (n=4).

SRBC in carp which were kept at 24° (Fig. 1). The first PFC could be detected at day 6. Peak response was reached on day 9 (93 ± 43 PFC/10^6 WC in pronephros). Following a secondary injection 1 month after the first, PFC were already observed on day 3. The peak of the response on day 8 was a fifty-fold higher than in the primary response (4800 ± 2360 PFC/10^6 WC in pronephros). PFC numbers diminished in the period after day 8 but up to 20 days after injection PFC were observed.

To assess peak primary and secondary anti-SRBC response in carp which were kept at 20°, animals were tested on a few selected days (Fig. 2). The peaks of both primary and secondary response were reached around 12 days after injection. The secondary injection gave rise to only ten times more PFC (95 ± 28 and 1104 ± 582 PFC/10^6 WC in pronephros, respectively).

In carp kept at 18°, the first PFC appeared in spleen, pronephros and mesonephros 11 days after primary injection with 10^9 SRBC i.m. (Fig. 3). Peak response was reached on day 17 (379 ± 185 PFC/10^6 WC in pronephros). Afterwards PFC numbers rapidly declined but PFC remained present in pronephros up to 30 days after injection. When a secondary injection was given 45 days after primary this did not result in a significant higher peak response in mesonephros or pronephros (304 ± 101 PFC/10^6 WC), although first PFC appeared 3–4 days earlier and remained for a longer period. Peak secondary response was reached in spleen and mesonephros on day 15, in pronephros on day 17.

At 16°, maximum numbers of PFC could be detected in spleen, pronephros (131 ± 29 PFC/10^6 WC) and mesonephros at around 27 days after injection (Fig. 4).

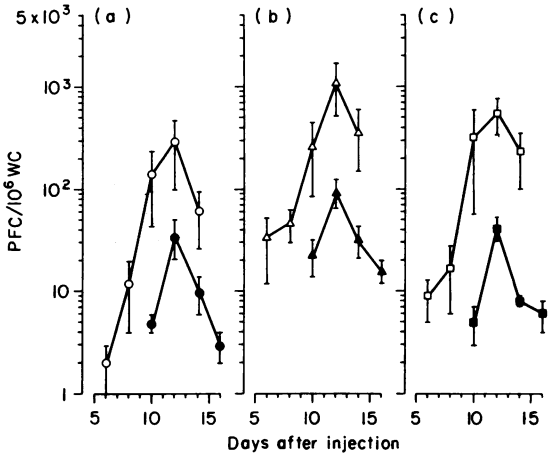

Figure 2. Kinetics of primary (filled symbols) and secondary (open symbols) anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 20°. Animals were injected twice with 10^9 SRBC. The interval between two i.m. injections was 1 month. PFC/10^6 WC = plaque-forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 SE (n=4).

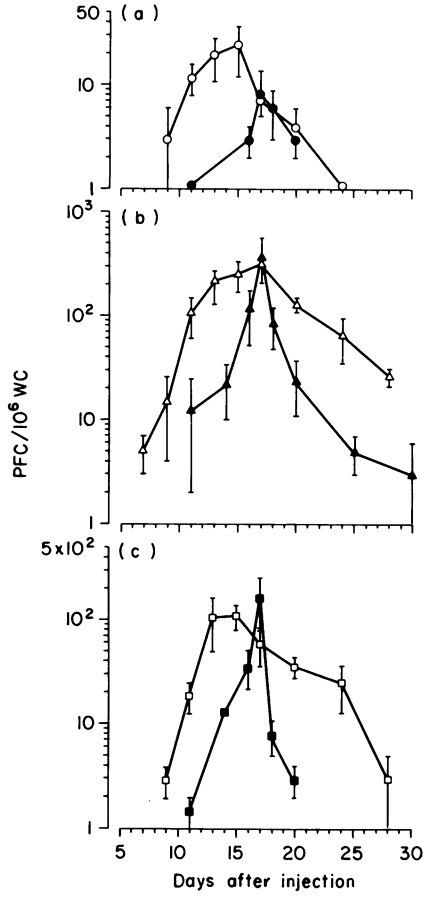

Figure 3. Kinetics of primary (filled symbols) and secondary (open symbols) anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (18 months old) kept at 18°. Animals were injected twice with 10^9 SRBC. The interval between two i.m. injections was 45 days. PFC/10^6 WC = plaque-forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 SE ($n=4$).

Peak primary response was reached around day 49 in carp kept at 12° (91 ± 35 PFC/10^6 WC in pronephros; Fig. 5).

To ascertain the observation that carp are able to develop PFC at low temperatures, PFC slides were incubated at 16 or 12° (the ambient temperature of the animals considered), instead of the usual 25° for plaque incubation. It appeared that at both 16 and 12° plaques were formed. However, the speed of development was low and attained levels did not meet values obtained with the 25° incubation (Table 2). It is concluded that antibody production and secretion continues at low temperatures, but at a reduced rate.

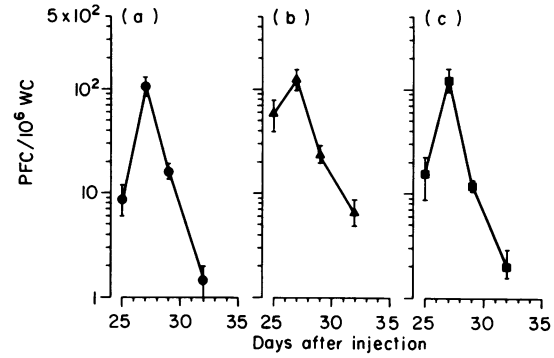

Figure 4. Kinetics of primary anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 16°. Animals were i.m. injected with 10^9 SRBC. PFC/10^6 WC = plaque-forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 SE ($n=4$).

In summarizing the results, it can be seen that with lowered temperatures the peak of the primary response is delayed (Fig. 6) but the magnitude of the response in unaltered. The anamnestic character of the secondary response, however, is gradually lost with lowered water temperatures.

DISCUSSION

The role of the pronephros in antibody production in teleost fish has been demonstrated in a number of studies (Smith, Potter & Merchant, 1967; Chiller, Hodgins, Chambers & Weiser, 1969; Chiller, Hodgins & Weiser, 1969; Pontius & Ambrosius, 1972; Sailendri & Muthukkaruppan, 1975). In the toad *Bufo marinus*, plasma cells were most abundant in intertubular lymphoid tissue of the kidney after injection with bovine serum albumin (BSA) (Cowden, Dyer, Gebhardt & Volpe, 1968). It was suggested that also in other lower vertebrates the kidney might be the major site for antibody production. However, in the tortoise *Agrionemys horsfieldii*,the kidney played no role in antibody production after immunization with SRBC (Rothe & Ambrosius, 1968). In rainbow trout (*Salmo gairdneri*) PFC were found in mesonephros after immunization with O-antigen of the bacterium *Yersinia ruckeri*, but numbers were not as high as in spleen (Anderson, 1978). In perch (*Perca fluviatilis*) mesonephros contained lower PFC numbers than spleen and pronephros after injection with SRBC (Pontius & Ambrosius, 1972). In goldfish (*Carassius*

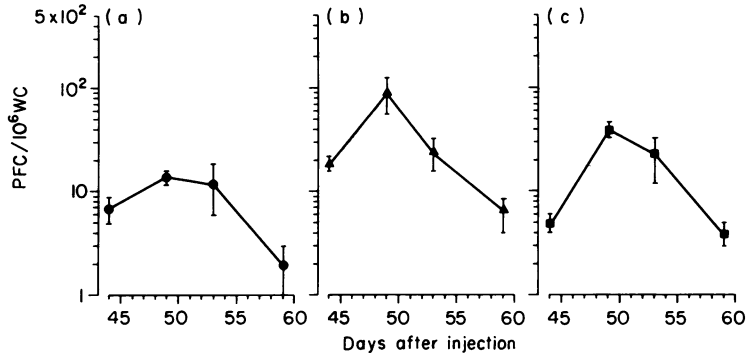

Figure 5. Kinetics of primary anti-SRBC response in spleen (a), pronephros (b) and mesonephros (c) of carp (6 months old) kept at 12°. Animals were i.m. injected with 10^9 SRBC. PFC/10^6 WC = plaque forming cells per 10^6 viable white cells. Each point represents the arithmetic mean ± 1 SE ($n = 4$).

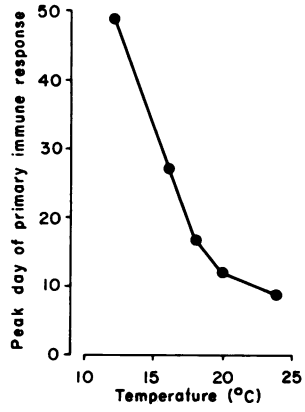

Figure 6. Temperature dependence of humoral anti-SRBC response in carp.

auratus) pronephros and mesonephros are major sites of antibody production against *Salmonella typhi* (Neale & Chavin, 1971). In our experiments with carp, high PFC numbers were found in mesonephros. This organ together with pronephros contributed 90% of the total PFC, illustrating the importance of the kidney as a whole for antibody formation.

In most higher and lower vertebrates the spleen is an important PFC-containing organ. In carp only low PFC numbers were found in spleen. However, it cannot be excluded that this organ plays an important role in the differentiation of B-like cells into plasma cells. Since PFC were observed in peripheral blood it is possible that proliferation and differentiation of B-like cells takes place in spleen and that maturing plasma cells migrate through the bloodstream and subsequently home in other organs (e.g. kidney). To verify this assumption splenectomy should be performed. Results of splenectomy experiments in other teleost fish are conflicting. Splenectomy in grey snapper (*Lutjanus griseus*) did not affect the antibody response

Table 2. Effect of incubation temperature on plaque development. Animals kept at 16° were tested for the presence of plaque-forming cells (PFC) in pronephros 25 days after an i.m. injection of 10^9 SRBC. Animals kept at 12° were tested 44 days after injection

Ambient temperature (°)	PFC incubation temperature (°)	PFC incubation time (h)	PFC/10^6 white cells in pronephros
16	25	2	61 ± 20*
16	16	5	26 ± 20
12	25	2	19 ± 3
12	12	5	4 ± 1
12	12	22	6 ± 1

* Arithmetic mean ± SE ($n = 4$).

against BSA (Ferren, 1967). Studies of Yu, Sarot, Filazzola & Perlmutter (1970) on the effect of splenectomy in blue gourami (*Trichogaster trichopterus*) suggest that the spleen is a major lymphoid organ in this species. Unfortunately splenectomy in carp is not possible because the spleen is interspersed with liver and pancreas and in most cases not a distinct organ but fragmented along the intestine.

Harris (1973) showed that dace (*Leuciscus leuciscus*) are capable of producing antibodies over their complete environmental temperature range (2–18°). The influence of temperature upon antibody production could be a general physiological effect as described for other metabolic processes (Huisman, 1974; Brett, 1979). A logarithmic relation between temperature and oxygen consumption or standard metabolism was observed by Brett (1976). In our experiments, it turned out that a linear relationship existed between peak day and low temperatures (12–18°) but the slope of the graph changed at 20° and higher (Fig. 6). The possibility of a general physiological effect of temperature on the immune response as a whole is therefore unlikely. In this respect, our results are in agreement with the ideas of Avtalion *et al.* (1976) and Matchalonis (1977) indicating that there are less and more temperature-sensitive steps in the immune response.

In all instances plaque assay slides were incubated at 25°. For carp kept at low temperatures (12–16°) this is well beyond their ambient temperature. To assure that carp were capable of performing an immune response at 12 and 16° PFC slides were also incubated at these temperatures. About 30% of the plaques were observed at low temperatures compared with the situation at 25°. It is admitted that a longer incubation period for plaque development was needed. It is possible that at 12° higher PFC numbers can be reached *in vivo* because our *in vitro* system was not devised for long incubation periods.

In contrast to Avtalion *et al.* (1970) who did not show antibody formation in carp at 12° using titration techniques, we were able to demonstrate a clear PFC response at this temperature (maximum day 49). Since no exponential relationship between temperature and peak day was observed and the magnitude of the response remained constant over the whole temperature range with carp of the same age, we expect that carp are able to mount an immune response at temperatures below 12°. However, in those cases lag periods will become very long and the protective effect of such a response is therefore questionable.

Criteria to assess immunological memory in vertebrates are an increased rate of antibody production and higher levels of antibody (Marchalonis, 1977). At 24°, a true secondary response was found, illustrated by an early appearance of the first PFC and a fifty times higher peak than in the primary response. At 20°, a ten times higher secondary response is reached on the same day as during the primary response. During the secondary response at 18°, the first PFC appear earlier but the peak coincides with the primary response. It must be mentioned that the animals were primed with high antigen doses (10^9 SRBC/animal) and that the interval between primary and secondary injection was about 1 month. Lower priming dose and prolonged interval between primary and secondary injection lead to a higher secondary response (Rijkers, Frederix-Wolters & van Muiswinkel, 1980b). Whereas the magnitude of the primary response is not affected by lower temperatures, the anamnestic character of the secondary response is gradually lost. This might indicate that the formation of memory cells is also a relatively temperature-sensitive process.

The relationship between temperature and peak day of the humoral immune response in carp (Fig. 6) closely matched the relationship between temperature and cellular immune response (allograft survival time) in goldfish (Hildemann, 1957). It is concluded that in bony fish humoral and cellular immunity are affected by temperature in a similar way.

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