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Stimulation of carp *Cyprinus carpio* lymphocytes *in vitro* by the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae)

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ABSTRACT: Interactions between extracts of adults or cercariae of the pathogenic blood fluke *Sanguinicola inermis* and lymphocytes of carp *Cyprinus carpio* were investigated *in vitro* by monitoring proliferation of pronephric and splenic lymphocytes at 10 and 20°C. Adult *S. inermis* were more mitogenic than cercariae at both temperatures. Splenic lymphocytes only responded to adult worm extracts at 10°C, whilst pronephric lymphocytes responded in a dose-dependent way to all extracts except those of cercariae at 10°C. Higher concentrations of the extracts reduced the degree of lymphocyte stimulation. These effects on pronephric and splenic lymphocytes of carp may be associated with stimulation and/or suppression of the immune response of the host.

KEY WORDS: *Cyprinus carpio* · *Sanguinicola inermis* · Lymphocyte · Mitogen

INTRODUCTION

Adult stages of the pathogenic fluke *Sanguinicola inermis* Plehn, 1905 inhabit the vascular system of carp *Cyprinus carpio* L. and shed eggs into the blood vessels (Lee 1990, Sommerville & Iqbal 1991, Kirk & Lewis 1993). Severe pathology has been associated with high intensities of adult parasites and, in particular, with eggs and migrating miracidia which become entrapped in host tissues and surrounded by inflammatory cells prior to encapsulation by collagen produced by fibroblasts (Lee 1990). Transmission electron microscopy has revealed that eggs less than 1 wk old were initially encapsulated by eosinophils, prior to infiltration of neutrophils and macrophages into the response around 1 to 2 wk old eggs. Eggs 2 to 3 wk of age were surrounded by several layers of macrophages, and the breakdown and destruction of the egg and enclosed miracidium occurred within a granulomatous lesion associated with eggs of 5 to 6 wk of age (Richards et al. 1994a).

Sanguinicola inermis infections also induce changes in the cellular composition of carp lymphoid organs (Richards et al. 1994b). The most marked of these changes was a significant reduction of erythrocytes in the spleen, which was attributed to parasite-induced haemorrhaging from capillaries. There were also significant increases of macrophages and neutrophils in the pronephros which correlated with the predominance of these cell types in the inflammatory response (Richards et al. 1994a). Richards et al. (1994b), however, found no evidence that lymphocyte numbers increased significantly in infected fish, although they postulated that lymphocytes may have increased in number relative to controls prior to 5 wk after the initial infection, which was when their first cell counts were made.

Previous studies have demonstrated that fish lymphocytes can be induced to proliferate *in vitro* in response to the presence of B-cell and T-cell mitogens such as phytohaemagglutinin A (PHA), concanavalin A (ConA), lipopolysaccharide of *Escherichia coli* (LPS) and lectin of pokeweed, *Phytolacca americana* (PWM) (Etlinger et al. 1976, Cuchens & Clem 1977, Liewes &

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van Dam 1982, Liewes et al. 1982, Warr & Simon 1983, Caspi et al. 1984, McKinney & Schmale 1988, Tillitt et al. 1988, Estepa & Coll 1992, Koumans-van Diepen et al. 1994) In addition, proliferation of lymphocytes isolated from fish lymphoid organs has been shown to be enhanced in the presence of extracts or antigens from bacteria such as *Vibrio anguillarum* (Yui & Kaattari 1987) and *Aeromonas salmonicida* (Pourreau et al. 1987, Tatner 1990) and the plerocercoid stage of the cestode *Ligula intestinalis* (Taylor & Hoole 1994).

The aim of this study was to determine whether extracts of adult and cercarial stages of *Sanguinicola inermis* induced proliferation of pronephric and splenic lymphocytes of carp. The results obtained were compared with those produced by mitogens known to stimulate mammalian B-lymphocytes and/or T-lymphocytes.

METHODS

Collection of parasite material. The snails *Lymnaea peregra* and *L. auricularia* were obtained from the margins of a lake in the West Midlands and screened for cercarial infections (Richards et al. 1994a). *Sanguinicola inermis* cercariae shed from these snails were used to prepare cercarial extracts or to infect carp and provide a source of adult flukes.

Carp, aged 3 to 4 mo, were obtained from a *Sanguinicola inermis*-free commercial fishery and exposed to 500 *S. inermis* cercariae (Kirk & Lewis 1992, Richards et al. 1994a). Carp were maintained at 20°C and adult flukes were recovered from the heart and associated vasculature from 3 to 5 wk post-infection (p. i.).

Preparation of parasite extracts. Extracts of up to 450 adults and 25 000 cercariae were prepared in ultrapure water by freeze-thawing each parasite suspension 5 times, sonicating on ice for 60 s, centrifuging at 16 500 × *g* at 4°C for 30 min to remove particulate matter and filter-sterilising the supernatant using 0.22 µm sterile filters. The protein concentration of each filtered supernatant was then determined using a Bicinchoninic acid assay kit (Pierce) The adult and cercarial extracts were diluted in sterile Leibovitz (L-15) culture medium supplemented with 5% heat-inactivated (20 min at 60°C) foetal calf serum (FCS), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), heparin (5 IU ml^{-1}) and L-glutamine (2 mM).

Mitogen preparations. Four mitogens, namely PHA (Sigma: Lot no. 92H95651), ConA Type IV-S (Sigma: Lot no. 83H9404), LPS of *Escherichia coli*: Serotype 055:B5 (Sigma: Lot no. 122H4025) and PWM (Sigma: Lot no. 53H9576) were prepared at 12 concentrations ranging from 0.975 to 1000 µg ml^{-1} in supplemented L-15 medium under sterile conditions.

Carp cell isolation. As found by other workers (e.g. Liewes & van Dam 1982, Liewes et al. 1982), attempts to isolate lymphocytes from carp organs gave inconsistent results, so the experiments were undertaken using mixed leucocyte suspensions.

Uninfected carp were bled by caudal puncture prior to removing the pronephros and spleen under sterile conditions. Each tissue was dissociated through a sterile stainless steel mesh (pore size 0.3 mm^2) into 1.2 ml of supplemented L-15 medium. The viability of cells in each suspension was tested by 0.4% w/v Trypan Blue exclusion throughout the experimental procedures. Cell counts were determined and fresh supplemented L-15 medium added to adjust the cell concentrations to 2.5×10^6 lymphocytes ml^{-1}. Cells were incubated at either 10 or 20°C for at least 1 h prior to the respective assays.

Proliferation assays. 100 µl of supplemented L-15 medium containing 2.5×10^5 splenic or pronephric lymphocytes from individual fish was added to wells of a 96-well plate. 100 µl of the respective mitogen or parasite extract suspension was then added to each well. In control wells, mitogen or parasite suspensions were replaced with 100 µl of supplemented L-15 medium. Plates were incubated in a humidified atmosphere at either 10 or 20°C for 72 h before addition of 0.5 µCi [C6-^{3}H]thymidine (Amersham: Lot no. B337) in 20 µl supplemented L-15 medium. After a further 24 h incubation, cells were harvested using a Titerex Cell Harvester (Flow Laboratories) by water lysis onto glass fibre filter paper (Flow Laboratories). Filter papers were transferred to scintillation vials (LIP), 4 ml of Ecoscint A scintillation fluid (National Diagnostics) was added and counts per minute (cpm) were recorded on a LKB Wallac 1219 Rackbeta Liquid Scintillation Counter.

Initial experiments were carried out at 20°C to determine the responsiveness of carp pronephric and splenic lymphocytes to the 4 mitogens at concentrations ranging from 0.48 to 500 µg ml^{-1}

Proliferation of lymphocytes from 4 uninfected carp was then compared at both 10°C and 20°C in the presence of extracts of *Sanguinicola inermis* adults or cercariae and the optimum concentrations of PHA, ConA, LPS and PWM. Statistical analyses were carried out on the original cpm data using Student's *t*-test.

RESULTS

The ability of each mitogen or parasite extract to stimulate lymphocyte proliferation was considered in terms of its Stimulation Index (SI), where:

$$SI = \frac{\text{cpm cells stimulated by parasite extract or mitogen}}{\text{cpm unstimulated control cells}}$$

Mitogen-induced stimulation of lymphocytes

Pronephric and splenic lymphocytes responded to both B-cell and T-cell mitogens. The highest mean SI values for pronephric lymphocytes were obtained with concentrations of PHA at 3.9 µg ml^{-1}, LPS at 62.5 µg ml^{-1} and both ConA and PWM at 0.45 µg ml^{-1} The highest mean SI for splenic lymphocytes were obtained with concentrations of PHA, ConA, LPS and PWM at 0.95 µg ml^{-1}. These mitogen concentrations were used in a subsequent experiment to compare mitogen SI with those SI produced by parasite extracts.

Lymphocyte stimulation by parasite extracts

The results indicate that *Sanguinicola inermis* extracts induced a differential lymphocyte proliferation response that was dependent on parasite stage, incubation temperature and the host lymphoid organ from which the cells were isolated.

Extracts of adult *Sanguinicola inermis* were more mitogenic than those of cercariae to pronephric lymphocytes at 20°C (Fig. 1; $t = 2.31$, $p < 0.05$) and 10°C (Fig. 1; $t = 3.38$, $p < 0.01$) and to splenic lymphocytes at 20°C (Fig. 2; $t = 3.88$, $p < 0.01$) and 10°C (Fig. 2; $t = 3.30$, $p < 0.01$).

Adult worm extracts were more mitogenic at 20°C than at 10°C to pronephric lymphocytes (Fig. 1a; $t = 7.26$, $p < 0.001$), but significantly less mitogenic at 20°C to splenic lymphocytes (Fig. 2a; $t = 8.76$, $p < 0.001$). In contrast, cercarial extracts were more mitogenic at 20°C than at 10°C to both pronephric lymphocytes (Fig. 1b; $t = 11.39$, $p < 0.001$) and splenic lymphocytes (Fig. 2b; $t = 15.12$, $p < 0.001$).

Adult worm extracts were more mitogenic to pronephric lymphocytes than splenic lymphocytes at 20°C ($t = 4.46$, $p < 0.001$), but not at 10°C ($t = 1.86$, $p > 0.05$). Cercarial extracts were more mitogenic to pronephric lymphocytes than splenic lymphocytes at both 20°C ($t = 5.10$, $p < 0.001$) and 10°C ($t = 6.55$, $p < 0.001$).

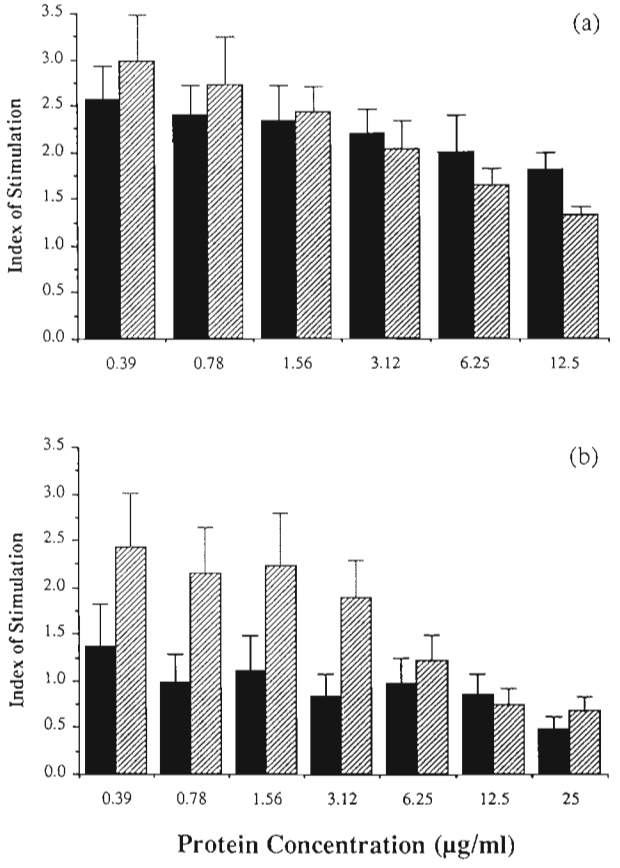

Fig. 1. Proliferation of pronephric lymphocytes of carp *Cyprinus carpio* in the presence of whole extracts of *Sanguinicola inermis* (a) adults and (b) cercariae at (■) 10°C and (▨) 20°C. Means + SEM

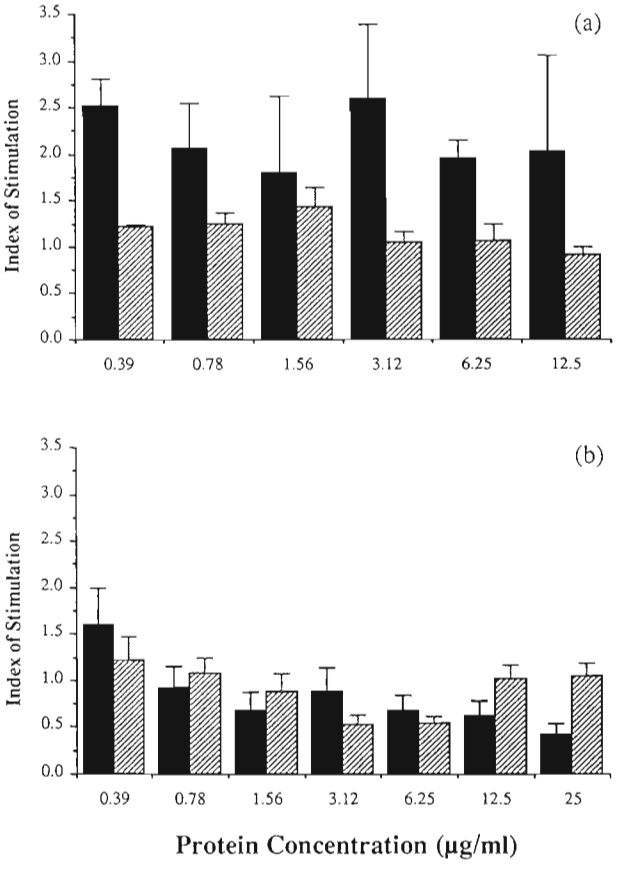

Fig. 2. Proliferation of splenic lymphocytes of carp *Cyprinus carpio* in the presence of whole extracts of *Sanguinicola inermis* (a) adults and (b) cercariae at (■) 10°C and (▨) 20°C. Means + SEM

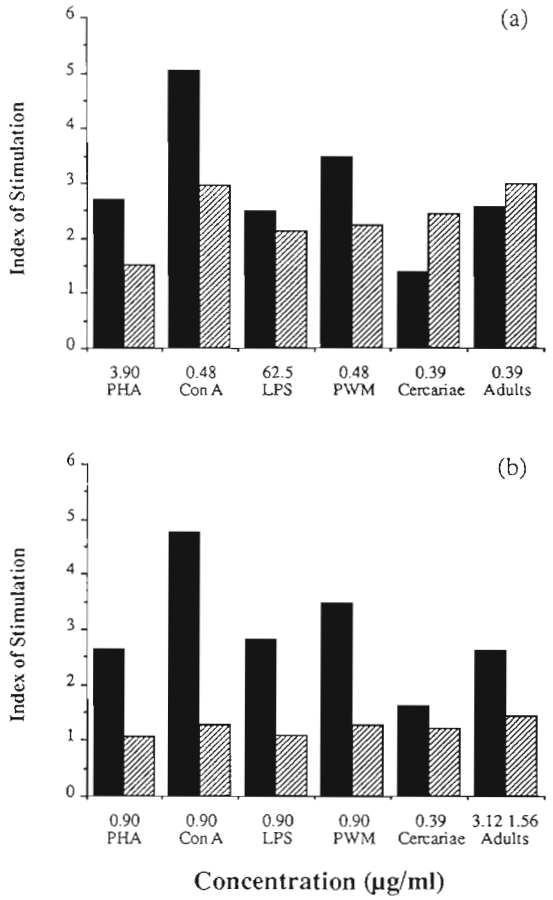

Fig. 3. Comparison between the highest levels of proliferation of (a) pronephric and (b) splenic lymphocytes of carp *Cyprinus carpio*, in the presence of the mitogens PHA, ConA, LPS and PWM, and whole extracts of cercariae or adults of *Sanguinicola inermis* at (■) 10°C and (▨) 20°C

Pronephric lymphocytes responded to *Sanguinicola inermis* adult and cercarial extracts in a dose-dependent way: higher concentrations of extracts reduced the degree of lymphocyte stimulation (Fig. 1). Splenic lymphocytes only responded to *S. inermis* adult worm extracts at 10°C (Fig. 2).

The highest mean SI values obtained for pronephric and splenic lymphocytes in the presence of adult and cercarial extracts were found to be comparable with those produced by the B-cell and T-cell mitogens (Fig. 3).

DISCUSSION

It has previously been established that carp elicit a cellular immune response against the eggs of *Sanguinicola inermis* which comprises eosinophils, neutrophils and macrophages (Richards et al. 1994a). The present study has demonstrated that extracts of both adult and cercarial stages of the parasite can also stimulate the proliferation of carp lymphocytes *in vitro*, in a dose-dependent manner. Previous observations have revealed that these blastogenic responses can be induced in mammalian lymphocytes *in vitro* by substances produced by other helminths such as the cestodes *Echinococcus granulosus* (Dixon et al. 1978) and *Taenia multiceps* (Rakha et al. 1991) and in pronephric lymphocytes of roach *Rutilus rutilus* by extracts of *Ligula intestinalis* (Taylor & Hoole 1994).

In the present study, marked differences were observed between the proliferative responses of carp lymphocytes that were related to the temperature at which the cells were incubated, the host organ from which they were isolated and the stage of the parasite to which they were exposed. The degree of stimulation was found to be comparable with levels produced by known T-cell and B-cell mitogens, although the possible antigen(s), either excretory-secretory or somatic, responsible for inducing the proliferative responses of lymphocytes from the pronephros and spleen, and their concentrations in the extracts are not known. The concentrations of parasite antigens that induced the proliferative responses may, therefore, have been much lower than those of the mitogens. In addition, it should be noted that antigens inducing the proliferation responses may not have been present at the same concentrations in adult and cercarial worm extracts and that the mechanisms of lymphocyte activation differ for mitogens and antigens.

Lymphocyte proliferation was found to be enhanced in response to both adult and cercarial extracts at 20°C, when compared with those at 10°C, and adult flukes were significantly more mitogenic than cercariae at both temperatures. This temperature-dependent effect may play an important role in this host-parasite interaction and could be related to the development and rate of maturation of adult *Sanguinicola inermis*. Adults can overwinter in infected carp in natural systems at relatively low temperatures (Lee 1990), whereas they mature after 2 to 3 mo in carp maintained at 15 to 18°C (Sommerville & Iqbal 1991) and in less than 1 mo in carp maintained at 20°C (Kirk & Lewis 1992). At this latter temperature, numbers of adult flukes peak around 30 d p.i. and the adults may only survive for up to 8 wk p.i. (Lee 1990). A reduction in the efficiency of the immune response at lower temperatures may explain the ability of adult *S. inermis* to survive in carp for relatively long periods over winter. The effects of this temperature-dependence may subsequently be reversed during the summer months as water temperatures rise to levels that enhance antibody production.

Temperature has previously been shown to play an important role in the effective functioning of the

immune response of fish (Avtalion et al. 1980, Rijkers et al. 1980, 1981, Clem et al. 1984). For example, Rijkers et al. (1980, 1981) found that numbers of primary antibody-producing plaque-forming cells (PFC) in carp maintained at 24°C peaked 9 d after injection with sheep red blood cells (SRBC), compared with an equivalent peak at 49 d post-injection in fish kept at 12°C. Similarly, Wishkovsky & Avtalion (1987) found a typical primary antibody response after injecting carp maintained at 25°C with formalin-killed *Vibrio anguillarum*, but not in fish injected with the same antigen and held at 12°C. It has been suggested from studies on mitogen-induced proliferation of fish leucocytes that temperature-related differences in antibody responses result from a reduced ability of helper cells to process antigens at lower temperatures rather than an inability of antibody-producing cells to produce antibody (Avtalion et al. 1976, Cuchens & Clem 1977, Liewes et al. 1982), or because accessory or antigen presenting cells such as monocytes and macrophages function less efficiently (Vallejo et al. 1992).

Whether this is the case in *Sanguinicola inermis* infections is not known. However, the reduced ability of carp lymphocytes to proliferate in response to *S. inermis* extracts at the lower temperature may suggest that the immune system is impaired. This may be an advantage to the parasite, particularly during the spring and autumn months, when peaks in cercarial release from *Lymnaea* spp. snails occur (Lee 1990) and water temperatures are lower. The vulnerable stages of the parasite occur during penetration and migration through the skin, subcutaneous tissues and vascular system at a time of reduced immune capability. Parasite survival may be enhanced by avoiding the immune responses. The host may, however, still be capable of responding to the presence of the parasite by utilising non-specific cellular responses. This has previously been found in studies on carp lymphoid organs that noted higher numbers of granulocytes in fish maintained at lower temperatures, which suggested that non-lymphoid defence may play a more important role when circumstances are less favourable for the formation of antibodies (Rijkers et al. 1981).

The fact remains, however, that *Sanguinicola inermis* can survive and reproduce effectively in carp over a wide range of environmental temperatures. Therefore, even if, as suggested, the humoral immune response is initiated against *S. inermis*, the parasite may have one, or more, as yet unknown mechanisms of evading the antibody responses. M. Roberts & D. Hoole (unpubl. data) have found that host molecules occur on the surface of adult *S. inermis*, but the nature of these molecules has not yet been determined. *S. inermis* may, therefore, be able to acquire host molecules and express them on its surface. This evasion strategy has previously been demonstrated by mammalian schistosomes (Capron & Camus 1979). Another evasion strategy used by parasites is the production of cytotoxic substances such as proteinase inhibitors, which suppress the effective functioning of the host immune system, and thus enhance parasite survival (Capron & Camus 1979, Hoole & Arme 1982, 1983, Pourreau et al. 1987, Taylor & Hoole 1989). Previous studies by Richards et al. (1994a) also found lysis of host cells around the eggs of *S. inermis*, but it is not known if this lysis occurred as a consequence of substances released from the entrapped eggs, which were subsequently encapsulated within a granulomatous lesion.

The present study demonstrated that at 20°C, most proliferation occurred in pronephric lymphocytes, with relatively little response being induced in splenic cells. At 10°C, however, lymphocytes from both the spleen and pronephros were stimulated, but to a significantly lesser degree than those of the pronephros at 20°C. Previous observations on changes in the cellular composition of the lymphoid organs of *Sanguinicola inermis*-infected carp maintained at 20°C have found relatively few changes in the leucocyte composition of the spleen, whereas in the pronephros there were marked and significant increases of leucocytes, particularly neutrophils (Richards et al. 1994b). Because the response of the pronephros was significantly greater than that of the spleen to both adult and cercarial extracts, particularly at 20°C, it is likely that the pronephros is more important in the immune response at higher temperatures, whilst the spleen, which responds to adult flukes at 10°C, may be of greater importance at lower temperatures.

The differential effect of *Sanguinicola inermis* extracts on the stimulation of lymphocytes isolated from the spleen and pronephros may reflect the heterogeneity of the respective lymphocyte populations. It seems likely, from studies on the ultrastructure and mitogen responses of carp lymphocytes, that at least 2 distinct subpopulations exist that respond differentially to the T-cell mitogens PHA and ConA and the B-cell mitogen LPS (Caspi et al. 1984). Furthermore, Koumans-van Diepen et al. (1994), using immunocytochemical techniques and monoclonal antibodies to identify lymphocytes isolated from carp blood, concluded that LPS stimulates surface immunoglobulin positive cells and PHA stimulates surface immunoglobulin negative cells. However, these 2 lymphocyte subpopulations are unlikely to be equally divided between the pronephros and spleen. Rijkers et al. (1980) found that 90% of the antibody-producing PFC against SRBC in carp were found in the pronephros and mesonephros, with only low numbers found in the spleen. Also, Liewes et al. (1982) found that both splenic and pronephric leucocytes of carp responded to

the T-cell mitogen PHA, and at very low levels to ConA, spleen leucocytes responded best to PWM but the B-cell mitogen LPS produced little stimulation.

The results of the present study may indicate that *Sanguinicola inermis* extracts are differentially stimulating the lymphocyte populations of the pronephros and spleen. It is suggested that at higher temperatures which prevail during the summer months, humoral immune responses, possibly mediated by pronephric lymphocytes, may be more important in the host response, whereas at lower temperatures non-specific responses, possibly mediated by T-cells in the spleen, may predominate.

Richards et al. (1994b) found no increases in numbers of lymphocytes in the spleen and pronephros of infected carp at 5 and 9 wk p.i., but the present study indicated that *Sanguinicola inermis* can induce differential lymphocyte proliferation. It is still unclear whether or not the parasite induces an antigen-specific antibody response. Further investigations are required to determine if *S. inermis* is stimulating B-cells and/or helper T-cells, or whether stimulation is of cytotoxic T-cells, or others that mediate in the cellular response by directly stimulating eosinophils, neutrophils and macrophages.

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