Effects of *Bothriocephalus acheilognathi* on the polarization response of pronephric leucocytes of carp, *Cyprinus carpio*

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Abstract

An *in vitro* assay was used to examine the effect of *Bothriocephalus acheilognathi* Yamaguti, 1934 (Cestoda: Pseudophyllidea) on the polarization response of pronephric leucocytes of carp, *Cyprinus carpio*. Leucocytes, isolated from naive, naturally-infected fish and carp injected intraperitoneally with cestode extracts, were exposed to parasite extracts (protein concentrations 0–10.0 $\mu g\,ml^{-1}$), for up to 24 h in the presence or absence of carp serum. In general, polarization responses of the pronephric leucocytes, primarily neutrophils and eosinophils, increased with incubation time although there was no significant difference in the response induced by the different protein concentrations. Differences in the polarization response were, however, observed in naive, naturally infected and injected fish and the cells responded differently in the presence and absence of carp serum. In the absence of carp serum the polarization response of pronephric leucocytes *in vitro* was significantly reduced with cells obtained from injected and naturally infected fish compared with those obtained from naive carp. This suppression of leucocyte migration was however reduced by the addition of carp serum to the *in vitro* system. The role of this interaction between the possible suppression of polarization induced by the parasite and stimulation by serum is discussed.

Introduction

The migration of leucocytes towards the site of infection or injury, which is a major component of the inflammatory response, can be identified by their change of shape from spherical to a characteristic polarized form (Zigmong *et al*., 1981). Factors that are chemoattractants for fish leucocytes can be host- and/or pathogen-derived. Of the host-derived factors, serum components (Newton *et al*., 1994), lipoxygenase products (Sharp *et al*., 1992) and some cytokines, e.g. IL8 (Van Damme, 1994) which has recently been sequenced in the lamprey, *Lampetra fluviatilis* (Najakshin *et al*., 1999) are potent chemoattractants. In addition, extracts of several species of fish helminths are chemoattractive and can apparently induce chemokinesis and chemotaxis of fish pronephric leucocytes (Sharp *et al*., 1991; Taylor & Hoole, 1993; Richards *et al*., 1996). To date however, the effect of an intestinal adult tapeworm on leucocyte migration from the pronephros has not been ascertained.

The cestode, *Bothriocephalus acheilognathi* Yamaguti, 1934 (formerly *B. gowkongensis*) is causing concern due to its pathogenicity and worldwide distribution. Since its recognition in the 1950s as an important disease in grass carp, *Ctenopharyngodon idellus*, the major fish species in China's aquaculture industry (Liao & Shih, 1956),

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extensive studies have been carried out on this cestode. However, relatively little is known about the relationship between the fish immune response and *B. acheilognathi* infections, although inflammation occurs in infected intestines and leucocytes have been noted on the surface of the parasite (Hoole & Nisan, 1994). Recent studies have revealed that parasite extracts increase antibody production and pronephric antibody producing cells in injected fish (Nie & Hoole, 1999) and stimulate proliferation of pronephric lymphocytes *in vitro* after 5 and 10 days post-injection (Nie *et al*., 1996). The present study was carried out to examine the effect of *B. acheilognathi* extracts on carp pronephric leucocyte polarization, and to establish whether there is any difference in the responses in naive, naturally-infected fish and fish injected with the worm extract.

Materials and methods

Parasite sources and extracts

The cestode, *B. acheilognathi*, was collected both from China and in the UK as described by Nie *et al*. (1996). In China, the worm was obtained from a cyprinid fish, *Hemiculter lusciculus* from Xingyun Lake in Yunnan province, southwest China. In the UK, the parasite was obtained from carp, *Cyprinus carpio* caught locally (Dagfield Pool, Cheshire). Cestodes from the two sources were mixed and the parasite extracts prepared as described by Nie *et al*. (1996).

Experimental design

Three treatments on carp were conducted. *Cyprinus carpio*, about six months old and 5.7–6.4 cm in fork length from a *B. acheilognathi*-free source within the UK (Fair Fisheries, Shropshire), were acclimatized in aerated dechlorinated tapwater at 20°C and fed on commercial fish food daily for 7 days prior to experimentation.

To investigate the effects of parasite extract on leucocyte polarization on cells previously exposed to the parasite antigens, four fish, maintained in 40 l of dechlorinated water at 20°C, were injected intraperitoneally with 1 ml cestode homogenates (protein concentration = 0.55 mg ml^{-1} saline) and the pronephros removed 8 days later. In addition, the effects of the parasite extracts were also examined on pronephric leucocytes isolated from four naive, uninfected fish and four naturally infected specimens (4.8–5.8 cm in fork length) that had been acclimatized at 20°C in the aquarium for at least 10 days prior to experimentation. Confirmation of infection was carried out by dissection of the intestines with 6, 23, 52, and 164 cestodes, being recovered from each fish respectively. The naturally infected carp were in the same year class as those from the cestode-free source.

Pronephric leucocyte suspensions

To reduce erythrocyte contamination of the cell suspension, freshly killed fish were bled by severing the tail at a location midway between the anal fin and the base of the caudal fin. The pronephros of each fish was removed into separate petri dishes containing 1.0 ml L-15 culture medium supplemented with 100 iu ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Nie *et al*. 1996), and disrupted through a sterile stainless steel mesh (pore size 0.3 mm). The cell viability in each suspension, which was determined using a trypan blue exclusion method, was always greater than 95%. The cell concentration was adjusted to 2.5×10^4 leucocytes ml^{-1} with culture medium.

Polarization assay

Samples of 100 μl pronephric leucocyte suspension and an equal volume of the appropriate concentration of parasite extract (0, 0.1, 1.0 and 10 μg protein ml^{-1} in culture medium) were added to wells of a flat-bottomed 96-well plate. To determine if the presence of carp serum influenced the polarization responses, wells were set up as above and heat-inactivated (20 min at 60°C) serum pooled from infected and uninfected fish was added at a concentration of 1%. Plates were incubated at 20°C for up to 24 h. At intervals from 0.5 to 24 h, cells were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and the percentage of the cells polarized in each well was determined using light microscopy by counting more than 200 cells. To determine which leucocyte types were undergoing polarization, cells were pelleted by centrifugation at 604 *g*, washed in buffer and post-fixed in 1% osmium tetroxide and processed for routine transmission electron microscopy (Richards *et al*., 1996).

Statistical analysis

Percentage values for polarized leucocytes were arcsine transformed for analysis of variance, and differences between treatment means at each time point were analysed using the t test.

Results

In general, polarization responses of pronephric leucocytes of *C. carpio* increased gradually with incubation time (fig. 1). The percentage of the cells polarized increased up to 12 h post exposure and remained high until 24 h. Statistical analyses indicated that such polarization responses were significantly different as the time of incubation increased even in the absence of parasite extract ($F = 236.92$, $P < 0.001$) and there was no significant difference in the polarization induced of pronephric leucocytes at the different protein concentrations used ($F = 0.34$, $P = 0.711$). The data for each protein concentration were therefore combined for naive, infected and injected fish in relation to the presence and absence of carp serum (fig. 1). Further analyses on this combined data revealed that significant differences exist in polarization responses of pronephric lymphocytes from naive, infected and injected fish ($F = 19.16$, $P < 0.001$), and such cells responded differently in the presence or absence of carp serum ($F = 3.99$, $P = 0.046$).

In the absence of carp serum (fig. 1A), the percentages of cells polarized 12 and 24 h after exposure were lower in those experiments where infected and injected fish were employed compared to naive fish ($P < 0.05$). Comparisons between the response of infected and injected fish revealed that the number of polarized cells was significantly lower ($P < 0.05$) in the latter treatment between 0.5 and 8 h post-exposure. In addition, the number of

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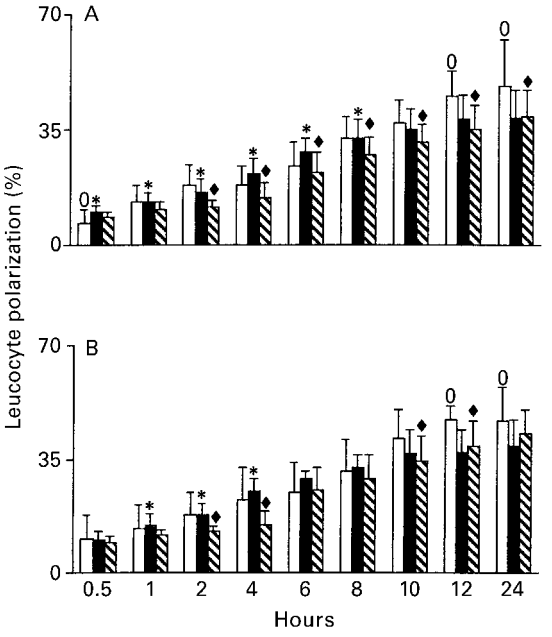

Fig. 1. Polarization response of pronephric leucocytes from naive (), naturally-infected (□) and injected (▨) *Cyprinus carpio*, induced by extracts of *Bothriocephalus acheilognathi*. A: in the absence of carp serum; B, in the presence of carp serum. Mean +/− SD. *, Significant difference ($P < 0.05$) between naturally-infected and injected carp; ♦, significant difference ($P < 0.05$) between naive and injected carp; ○, significant difference ($P < 0.05$) between naive and naturally-infected carp.

polarized cells was lower ($P < 0.05$) in injected fish compared to naive fish at time periods greater than 2 h post-exposure. In contrast, the presence of carp serum reduced the number of significant differences between the treatments (fig. 1B). Although a significant difference ($P < 0.05$) in the leucocyte polarization response occurred between naturally infected and naive fish after 12 h exposure to parasite extract, a significant reduction ($P < 0.05$) in the response of leuocytes from injected carp compared to infected fish only occurred between 1 and 4 h exposure. In addition, a significant reduction in the polarization response ($P < 0.05$) of leucocytes from injected fish compared to naive carp only occurred at 2, 4, 10 and 12 h exposure to parasite extract.

Ultrastructural examination revealed that the majority of cells undergoing polarization were neutrophils (fig. 2A) and eosinophils (fig. 2B). Both cell types comprised a typical polarization form with the nucleus located at one pole of the cell and a pseudopodial cytoplasmic extension occurring at the leading edge.

Discussion

Hoole & Nisan (1994) have previously observed leucocytes including macrophages, eosinophils and lymphocytes at the site of infection of *B. acheilognathi* in intestines of *C. carpio*, and suggested that these cells may migrate through the intestinal epithelium and occur on the worm surface. Liao & Shih (1956) and Bauer *et al*. (1973) also revealed an infiltration of leucocytes into the intestinal wall of the cyprinid fish infected with *Bothriocephalus*. The induction of polarization in pronephric leucocytes, primarily neutrophils and eosinophils, isolated from naive, infected and injected fish in the absence of carp serum may indicate that *B. acheilognathi*-derived molecules are chemoattractive for pronephric leucocytes. Richards *et al*. (1996) also found that carp neutrophils and eosinophils were induced to polarize by live *Sanguinicola inermis*. The relatively low percentage of the leucocytes undergoing polarization on exposure to extracts of *B. acheilognathi* may reflect the low protein concentrations used. Other studies on fish leucocyte migration have employed various ranges of protein concentrations. For example, Taylor & Hoole (1993) used 5 and 10% extracts of plerocercoids of *Ligula intestinalis*, whilst other workers have either used higher protein concentrations, e.g. up to 100 $\mu g\,ml^{-1}$ for plerocercoid extracts of *Diphyllobothrium dendriticum* or entire parasites as sources of chemoattractants (e.g. Sharp *et al*., 1991; Richards *et al*., 1996).

It is interesting to note that leucocytes isolated from naive, infected and injected *C. carpio* have significantly different polarization responses to *B. acheilognathi* extracts. For fish infected with *B. acheilognathi*, the polarization response was lower than that for cells from naive fish after 12 h exposure to parasite extract and was not affected by the addition of serum. In contrast, the polarization response of pronephric leucocytes from injected fish was significantly reduced compared to cells from naturally-infected and naive carp up to 10 h and after 2 h exposure to parasite extract respectively. This may suggest that the parasite is producing a factor(s), which suppress the polarization response of pronephric leucocytes in carp either infected with the cestode or injected with *Bothriocephalus* extracts. In a previous study, Nie *et al*. (1996) injected carp with *B. acheilognathi* extracts and found that, although proliferation of pronephric leucocytes was significantly greater in injected fish compared with controls, this difference was not observed at 10 days post-injection. They suggested that the response of fish injected with the cestode extract might have decreased by 10 days post-injection and immunosuppression might have occurred. In the present study, the fish had been injected 8 days previously with the same parasite protein concentration (0.55 $mg\,ml^{-1}$) and leucocytes exposed to a similar parasite protein concentration range *in vitro* (0.1–10 $\mu g\,ml^{-1}$) as that used by Nie *et al*. (1996). The lower polarization response induced in the absence of carp serum supports the view that immunosuppression may occur when the fish is injected with the cestode extract. That this suppression of the polarization response of leucocytes is greater with cells from injected fish compared to naturally-infected individuals may reflect the route and amount of exposure of the leucocytes to the parasite antigen. It is interesting to note, however, that this difference is lost the longer the cells are exposed to parasite extract *in vitro*, i.e. greater than 12 h.

The addition of host serum to the cultures resulted in an increase in leucocyte polarization and a reduction in the number of time periods where suppression of the parasite-induced polarization was observed. Similar observations have been obtained in other fish/parasite systems. Taylor & Hoole (1993) observed an enhanced

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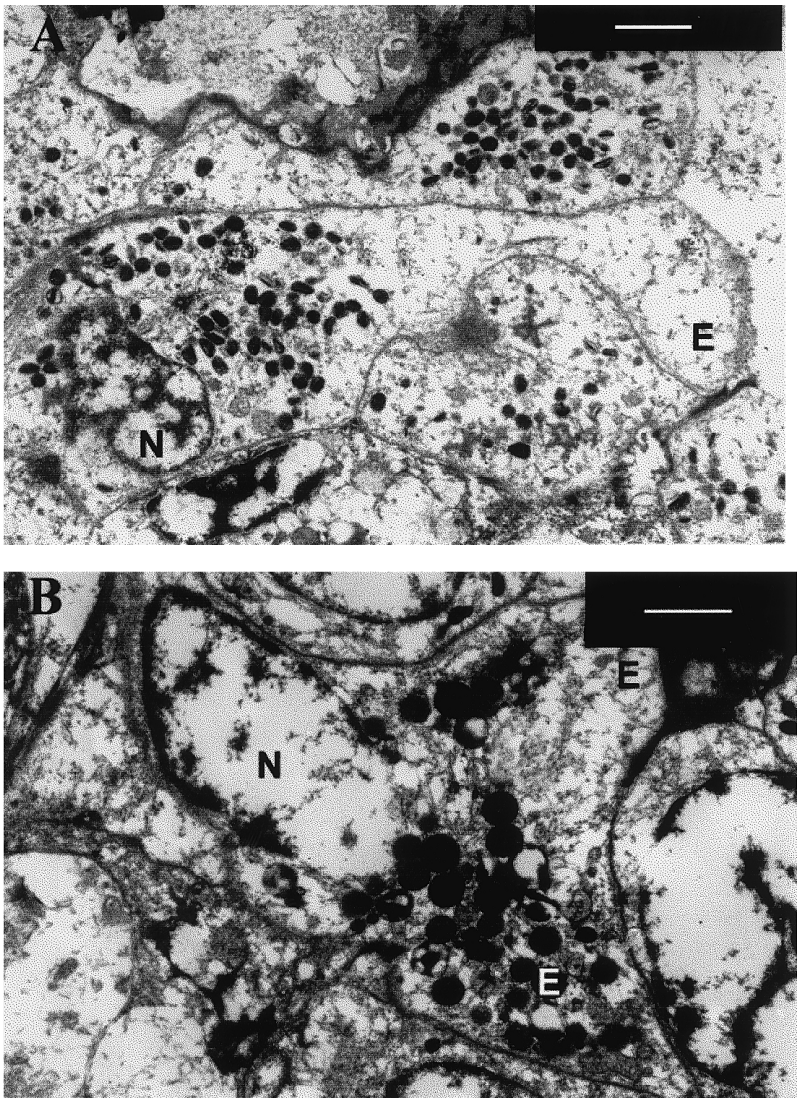

Fig. 2. Polarization of pronephric leucocytes of *Cyprinus carpio* exposed to extracts of *Bothriocephalus acheilognathi* in vitro. A, Neutrophil; B, eosinophil. In both cell types note elongation of leucocyte, polar position of nucleus (N) and leading migrating egde (E). Scale bar = 2 μm.

roach (*Rutilus rutilus*) polarization response of pronephric leucocytes when the culture medium was maintained at 20°C and supplemented with untreated and heat-inactivated fish serum. Richards *et al*. (1996) also found that either untreated or heat-inactivated carp serum enhanced the polarization of carp leucocytes incubated with cercariae of *S. inermis* between 0.25 and 3 h post-exposure. It has also been shown that the combination of host serum and bacterial-derived factors can significantly enhance migration of fish leucocytes (Lamas & Ellis, 1994; Newton *et al*., 1994). It is possible that factors in inflammatory exudate fluid (MacArthur *et al*., 1985) and leukotrienes (Hunt & Rowley, 1986) have a role in inducing the locomotion of leucocytes. The heat-inactivated carp serum used in this present study may thus have the same effect and account for the higher values of percentages observed. This may suggest that the damage induced by infection (Hoole & Nisan, 1994) may be an integral component in reducing the suppression of leucocyte migration possibly induced by the parasite.

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