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Role of cellular response in elimination of the monogenean *Neoheterobothrium hirame* in Japanese flounder *Paralichthys olivaceus*

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ABSTRACT: Adult worms of the blood-feeding monogenean parasite *Neoheterobothrium hirame*, which cause anemia in the Japanese flounder *Paralichthys olivaceus*, attach to the host fish by embedding their posterior part deeply into the host tissue. To investigate the possibility that cellular responses of the host fish can eliminate *N. hirame*, flounder were experimentally infected with *N. hirame* larvae and reared in either fed or starved conditions. Mature parasites were identified on the buccal cavity wall of the fish 33 d post-infection (Day 33). Monocytes/macrophages and granulocytes increased rapidly in the blood and infected sites after the appearance of mature parasites. These cells adhered to the tegument of the parasites. In addition, a few cells with large electron-dense granules (DGCs) were observed in the inflammatory foci. On Day 47, the tegument of some parasites collapsed partially and were phagocytosed by the infiltrated host cells. Some infiltrated cells adhered directly to the inner tissues of the parasites. On Day 54, in the fed fish group, the loss of the tegument led to damage of the parasites' inner tissue by a large number of infiltrated cells. In this group, the elimination of the parasites was noted from Day 47 to 54. These observations probably suggest that the cellular response of the host fish destructed the parasite's posterior part embedded in the tissue, thereby eliminating the parasites. On the other hand, a high mortality was observed in the starved group. The starved fish developed much more severe anemia than the fed fish, and the elimination of the parasites was not observed in this group. The results of the present study suggest that flounder can eliminate *N. hirame* if they are fed sufficiently.

KEY WORDS: Flounder · Leucocytes response · *Neoheterobothrium hirame* · Monogenean parasite

INTRODUCTION

Recently, severe anemia, identified as a hypochromic microcytic anemia accompanied by hypoglobulia and a structural abnormality of the erythrocytes, has been observed frequently in both wild and cultured Japanese flounder *Paralichthys olivaceus* in Japan (Miwa & Inouye 1999, Yoshinaga et al. 2000a, Nakayasu et al. 2002a). It has been suggested that the blood-feeding monogenean parasite *Neoheterobothrium hirame* is the causative agent (Yoshinaga et al. 2001, Nakayasu et al. 2002b). Immature worms of *N. hirame* attach to the gill filaments of the flounder with clamps, and mature worms migrate and infect the wall of the buccal cavity by embedding the parasite's posterior part which consists of the haptor and isthmus region, deeply into the host tissue (Ogawa 1999, Anshary & Ogawa 2001). The parasite first appeared on the Japanese flounder in 1993 and has since prevailed in the flounder in the waters surrounding Japan

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(Ogawa 1999). Commercial catches of wild flounder in the Sea of Japan decreased according to the prevalence of infection (see Ogawa 2002), suggesting that the decline in the flounder population is caused by a host mortality due to infection with *N. hirame*. In experimental challenges, high mortality accompanied by severe anemia was observed in the infected flounder (Yoshinaga et al. 2001). Nakayasu et al. (2002b) studied the hematology of anemic flounder experimentally generated by repeated bleedings in fed and starved conditions. The degree of anemia in the flounder was found to be greatly influenced by the nutritional conditions. Yoshinaga et al. (2001) showed that the anemia reproduced by experimental infections with *N. hirame* was less severe in fed fish than starved fish. Hence, prevention of the anemia by supplying sufficient nutrition may be an important factor for the survival of infected fish.

Wild flounder with scars in the buccal cavity wall, indicating previous infection by mature parasites, but devoid of the parasite have often been observed (Mushiake et al. 2001). The reason why these fish were able to eliminate the parasites, however, is unknown. Anshary & Ogawa (2001) discussed the possibility that the defense reaction of the host might have eliminated the parasites from the flounder. Adult parasites are directly attacked by the host's leucocytes since the parasite's posterior part is deeply embedded in the host tissue. In the host tissue surrounding the parasite, intensive infiltration of inflammatory cells and necrosis of host cells were observed (Anshary & Ogawa 2001). Nakayasu et al. (2003) also reported that the infiltrated cells, which consisted of macrophages, granulocytes and the cells with highly electron-dense granules (DGC), adhered to the tegument of *Neoheterobothrium hirame*. These infiltrated or adhered cells are likely to play important roles in the elimination of *N. hirame* in the flounder.

The aim of this study was to investigate whether experimentally infected Japanese flounder could eliminate *Neoheterobothrium hirame* when they were fed sufficiently. The cellular response that might be involved in the elimination process of the parasite was also investigated.

MATERIALS AND METHODS

Parasite. Japanese flounder infected with *Neoheterobothrium hirame* were reared in a tank in the National Research Institute of Aquaculture (NRIA). A nylon mesh bag (mesh opening: 108 µm) was placed at the outlet of the drain pipe of the tank so as to trap *N. hirame* eggs. The trapped eggs were collected once a day.

Fish. Healthy juvenile flounder were purchased from a private hatchery (Nisshin Marine Tech) where fish are cultured using water taken from a saltwater well and the occurrence of anemic flounder and *Neoterobothrium hirame* infection have not been recorded. They were reared in NRIA until the beginning of the challenge experiments.

Experimental design. Japanese flounder, weighing approximately 90 to 120 g, were used for the experimental infection with *Neoheterobothrium hirame*. Prior to their use in this experiment they were confirmed free of parasite infection. Two experimental sets were made. Each set consisted of a group of 30 fish in a 300 l tank and a group of 13 fish in a 150 l tank. In each 300 l tank, a nylon mesh bag (mesh opening: 108 µm) containing 1.0×10^4 worm eggs was suspended for 7 d for the challenge. In each 150 l tank, a group of 13 fish was reared as control group. Each tank was supplied with constant aeration and running seawater at 22°C. The fish in 1 experimental set were fed commercial pellet food at a rate of 2% body weight 5 times a week from 7 d after the start of the experiment (Day 7), while the groups in the other experimental set were reared without feeding. The water turnover rate was 10 times d^{-1} until Day 7. From Day 7, the rate was increased to 45 times d^{-1} until the end of the experiment in order to prevent a re-infection with *N. hirame* that might be reproduced in the experimental tanks.

On Day 21, 10 fish were randomly selected from each challenged group and transferred to each additional tank (150 l). These fish were reared in the same way as their original groups (either fed or starved) to monitor the infection rate and mortality. From Day 26 the fish in the 2 monitoring groups and the 2 control groups were macroscopically examined weekly for adult worms by illuminating the buccal cavity. Three fish were sampled for hematological and histological examination from the remaining fish of each challenged group on Days 0, 26, 40, 47 and 54. However, for the challenged and starved group, no sampling was performed on Day 54 because of the high mortality in this group. For the 2 control groups, 3 fish from each group were killed for blood collection on both Day 0 and Day 61, respectively.

Hematological examination. Blood was sampled with heparinized syringes from the caudal blood vessels of fish. Hemoglobin (Hb) concentration in the blood was determined by the cyanmethemoglobin method. The total number of leucocytes (peripheral blood leucocytes; PBLs) was counted in samples from the whole blood using a Bürker-Türk's counting chamber. PBLs were then separated from the whole blood by discontinuous density-gradient centrifugation using Percoll (Pharmacia) at a density of 1.077 g ml^{-1}. A preliminary experiment showed that all leucocyte types were successfully sepa-

rated at this density of Percoll. Separated PBLs from each fish were attached onto microscopic slides by centrifugation using Cytospin (Shandon). These slides were stained with May-Grünwald and Giemsa, and observed under a light microscope. The ratios of lymphocytes, granulocytes, and monocytes in PBLs were determined using these slides as described by Nakayasu et al. (2003). The flounder has only 1 type of granulocyte in the blood (Nakayasu et al. 2003). Moreover, since spherical-shaped thrombocytes are difficult to distinguish from lymphocytes, these cells were counted as lymphocytes. Each leucocyte count was calculated by multiplying the ratio of the leucocyte on the smear by the total PBL count.

Statistics. The data obtained from hematological examination were subjected to Student's *t*-test in order to detect significant difference between means. A value of $p < 0.05$ was considered to be significant.

Histology. The gills or buccal cavity wall with attached *Neoheterobothrium hirame* were excised from the challenged fish. Portions of the tissues were fixed in Davidson's solution and routinely embedded in paraffin. The samples were then sectioned at 4 µm and stained with hematoxylin-eosin. Portions of the buccal cavity wall were fixed in 2.5% glutaraldehyde, postfixed with 1% OsO_4, and dehydrated through an ethanol series. Subsequently, the samples were embedded in epoxy resin (Epon; TAAB Laboratories), ultra-thin sectioned, and stained with uranyl acetate and lead citrate, for electron microscopy (JEM-1010, JEOL).

RESULTS

Mortality and infection rate in experimental fish

The infection rate of *Neoheterobothrium hirame* and mortality in each group are shown in Table 1. Mature parasites were observed on the buccal cavity wall of the fish in both challenged groups on Day 33. After infection with mature parasites, high mortality was observed in the starved group. On the other hand, 6 out of 8 fish in the fed group that survived had eliminated the parasites by the end of the experiment.

Hematological examination

Anemia developed in both of the challenged groups after the migration of parasites to the buccal cavity wall. However, the starved fish developed much more severe anemia than the fed fish (Fig. 1). When the control groups were examined at the end of the experiment, no difference was observed in Hb concentrations between the fed and starved groups.

Changes in the number of each leucocyte population in the challenged groups are shown in Table 2. On Days 0 and 26, most of the PBLs in the challenged fish of both the unfed and fed groups were lymphocytes and thrombocytes. After the migration of parasites to the buccal cavity wall, the numbers of lymphocytes and thrombocytes sharply decreased, although the numbers of granulocytes and monocytes increased in the challenged fish. The decrease in the number of lymphocytes in the starved group was especially conspicuous.

Table 1. *Neoheterobothrium hirame* infecting *Paralichthys olivaceus.* Infection rate of the adult and cumulative mortality in the control and challenged groups of the Japanese flounder. Infected fish were identified by macroscopical examination for adult worms on the buccal cavity wall

	Day 40		Day 47		Day 54		Day 61	
	No. of fish infected/ survived	Mortality (%)	No. of fish infected/ survived	Mortality (%)	No. of fish infected/ survived	Mortality (%)	No. of fish infected/ survived	Mortality (%)
Starved								
Control	0/10	0	0/10	0	0/10	0	0/10	0
Challenge	10/10	0	6/6	40	1/1	90	1/1	90
Fed								
Control	0/10	0	0/10	0	0/10	0	0/10	0
Challenge	10/10	0	7/9	10	3/8	20	2/8	20

Sequential histological change in infected tissue with *Neoheterobothrium hirame*

On Day 26, immature worms that attached to the gill with clamps were found in all fish sampled from the challenged groups (Fig. 2), although no clear inflammatory response was observed. On Day 40, adult worms were noted on the buccal cavity wall of all fish sampled from the challenged groups. The number of adult worms found in these individuals varied from 5 to 25. The posterior part of the adult worms penetrated deeply into the buccal cavity wall where it induced an inflammatory response. The inflammation was observed in the connective tissue around the parasite. Electron microscopy showed the presence of leucocytes directly adhered to the parasite tegument. Identification of infiltrated and adhered cells was based on the morphological criteria described by Nakayasu et al. (2003). These cells mainly consisted of monocytes/macrophages and granulocytes. A few dense granular cells (DGCs)

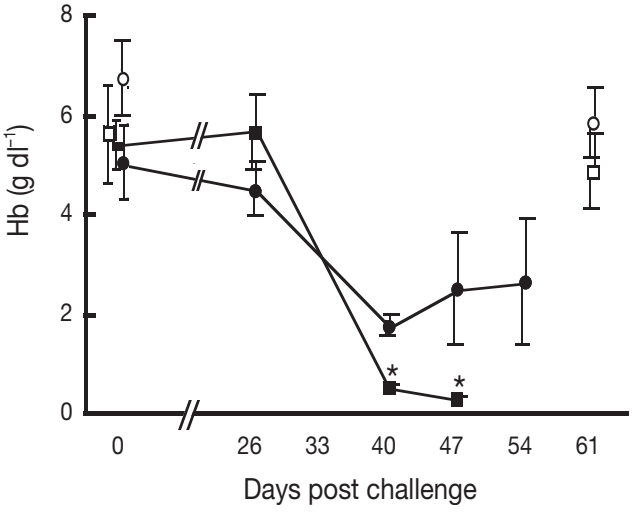

Fig. 1. *Neoheterobothrium hirame* infecting *Paralichthys olivaceus*. Changes in the concentration of Hb in the peripheral blood of flounder challenged with *N. hirame*. Mean values are plotted with the standard deviations. *: significant difference from the fed challenged group ($p < 0.05$). ■: starved and challenged group; ●: fed and challenged group; □: starved control group; ○: fed control group

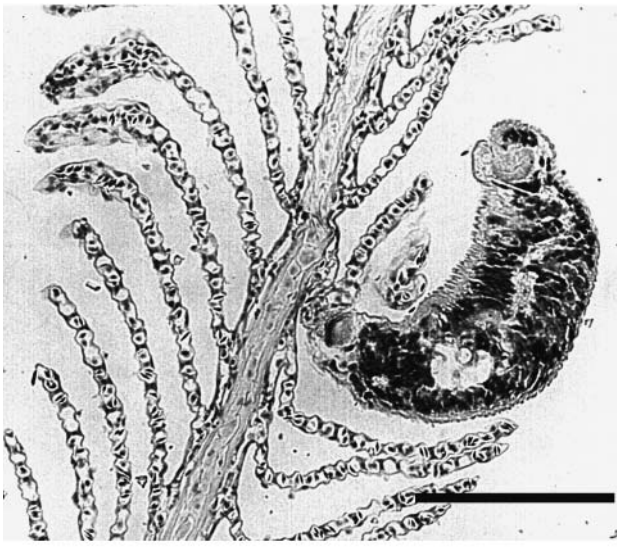

Fig. 2. *Paralichthys olivaceus* infected by *Neoheterobothrium hirame*. A histological section of the infected gill. An immature parasite attaching to the gill filament by means of clamps (H&E stain). Scale bar = 100 µm

were also observed in the infected sites. On Day 47, parasite clamps were observed in muscle and connective tissue where marked infiltration of inflammatory cells and fibroblasts had occurred (Fig. 3a). Transmission electron microscopy (TEM) observations revealed that the parasite body in the host tissue was surrounded by numerous infiltrated cells containing macrophages, granulocytes and DGCs (Fig. 3b). A vacuolar structure was often observed in the parasite's tegument adjacent to the adherent site of the leucocytes. Electron-dense substances, which were presumably released from the infiltrated cells, were often observed in the proximity of the tegument (Fig. 3c). Furthermore, the tegument of some parasites collapsed partially (Fig. 4a), and infiltrated cells adhered directly to the inner tissue of the parasite (Fig. 4b,c). Until this stage the cellular response in infected tissue was found to be qualitatively the same in both the fed and the unfed groups. On Day 54, in the tissue sections of the fed challenged fish, the posterior part of the parasite was invaded by a large number of infiltrated cells and its outline was no longer visible (Fig. 5a). At the ultrastructural level, inner tissues of parasites had collapsed at points where many infiltrated cells were observed (Fig. 5b). These tissues lost their structure completely and dead cell debris was frequently observed. In addition, elimination of the parasites was observed in 2 out of 3 fish

Table 2. *Parlichthys olivaceus* infected by *Neoheterobothrium hirame*. Changes in the concentrations of leucocyte populations in the peripheral blood of challenged flounder in the starved and fed group. Values are mean ($\times 10^6$) ± SD of 3 fish. *: significant difference from the value of Day 0 ($p < 0.05$)

		Number of cells (1 ml blood)$^{-1}$				
		Day 0	Day 26	Day 40	Day 47	Day 54
Starved challenged group	Lymphocyte	57.2 ± 18.3	40.7 ± 12.4	3.3 ± 1.0*	4.4 ± 3.2*	–
	Thrombocyte	19.8 ± 9.7	13.3 ± 8.2	0.6 ± 0.2*	0.9 ± 0.6*	–
	Granulocyte	2.1 ± 1.9	2.5 ± 2.3	8.7 ± 4.6*	28.1 ± 9.3*	–
	Monocyte	0.4 ± 0.5	0.3 ± 0.3	1.7 ± 0.6*	5.7 ± 1.1*	–
	Erythorcyte	3206.7 ± 306.6	3196.7 ± 468.2	1043.3 ± 60.2*	836.7 ± 119.3*	–
Fed challenged group	Lymphocyte	45.5 ± 14.6	51.8 ± 21.3	23.2 ± 7.0	14.1 ± 13.0*	28.8 ± 16.6
	Thrombocyte	14.5 ± 4.6	15.8 ± 8.2	7.3 ± 2.4	4.5 ± 4.1*	10.1 ± 5.5
	Granulocyte	3.5 ± 1.7	2.2 ± 2.4	9.0 ± 3.8	25.6 ± 8.0*	16.7 ± 18.1
	Monocyte	0.7 ± 0.4	0.3 ± 0.2	1.4 ± 1.1	4.6 ± 1.7*	2.3 ± 2.2
	Erythorcyte	3046.7 ± 360.9	2880.3 ± 235.2	1523.3 ± 103.1*	1781.3 ± 560.8*	1906.3 ± 571.3*

sampled from this group. The debris of the parasite body was observed in the buccal cavity wall of these fish. The remnants of the parasites were surrounded by infiltrated cells (Fig. 6a). Many macrophages and granulocytes were observed in these areas. These cells had well developed phagosomes containing tissue debris. Granulation tissue was formed around these areas (Fig. 6b).

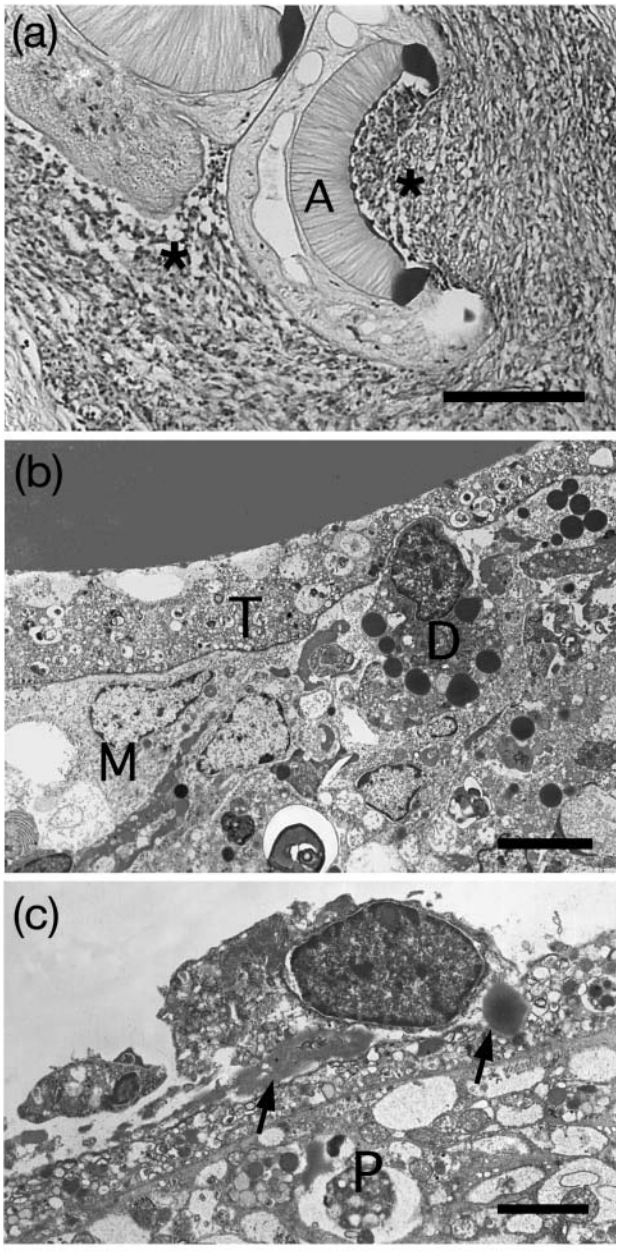

Fig. 3. *Neoheterobothrium hirame* infecting *Paralichthys olivaceus*. Light and electron micrographs of the infiltrated cells in close proximity to the surface of the parasite at 47 d post-challenge. (a) The attachment organ (A) of the parasite was surrounded by numerous infiltrated cells (*) (H&E stain). Scale bar = 100 µm. (b) Infiltrated cells including macrophages (M) and DGCs (D) on the tegument (T), forming multiple layers. Scale bar = 4 µm. (c) Electron-dense material (arrows) presumably released from infiltrated cells to the tegument of the parasite (P). Scale bar = 2 µm

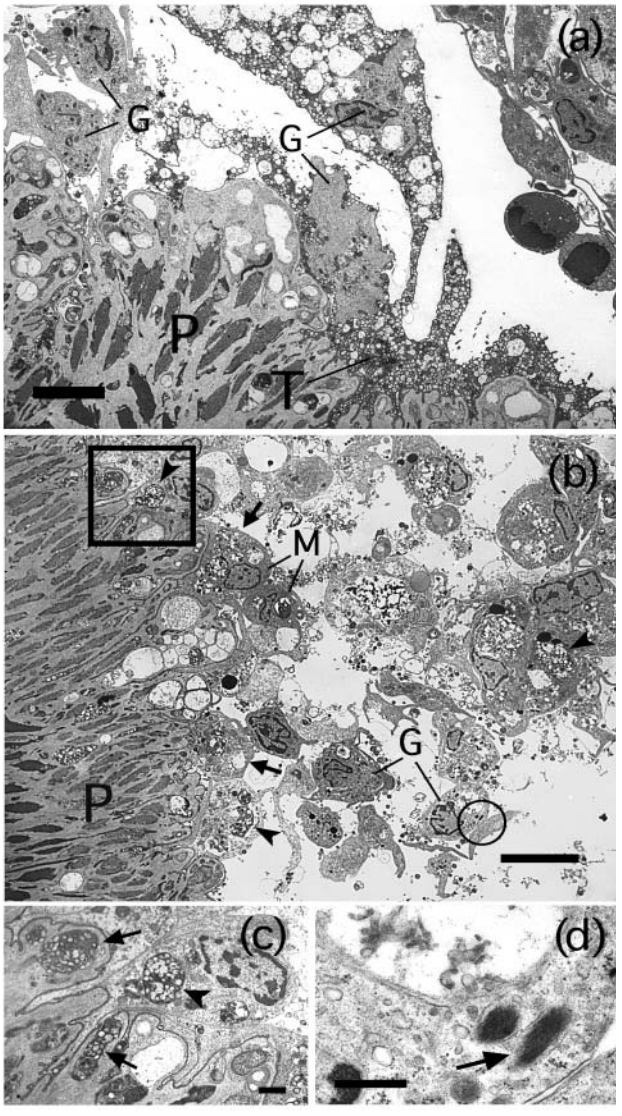

Fig. 4. *Neoheterobothrium hirame* infecting *Paralichthys olivaceus*. Transmission electron micrograph at 47 d post-challenge, showing the parasite (P) that lost the tegument. (a) Infiltrated granulocytes (G) were observed in the partially destroyed tegument (T). Scale bar = 4 µm. (b) Leucocytes directly adhere to the inner tissue of the parasite (arrows). Arrowheads indicate remnants of the tegument in phagosomes of macrophages (M) and granulocytes (G). Scale bar = 6 µm. (c) Micrograph enlargement of boxed area from (b), showing detail of the remnants of the tegument (arrowhead) in phagosome of macrophage. Arrows indicate the teguments which are being secreted from an inner tissue of the parasite. Scale bar = 1 µm. (d) Micrograph enlargement of circled area in (b). Granulocytes were characterized by granules having a fibrillar structure (arrow) in the cytoplasm. Scale bar = 0.5 µm

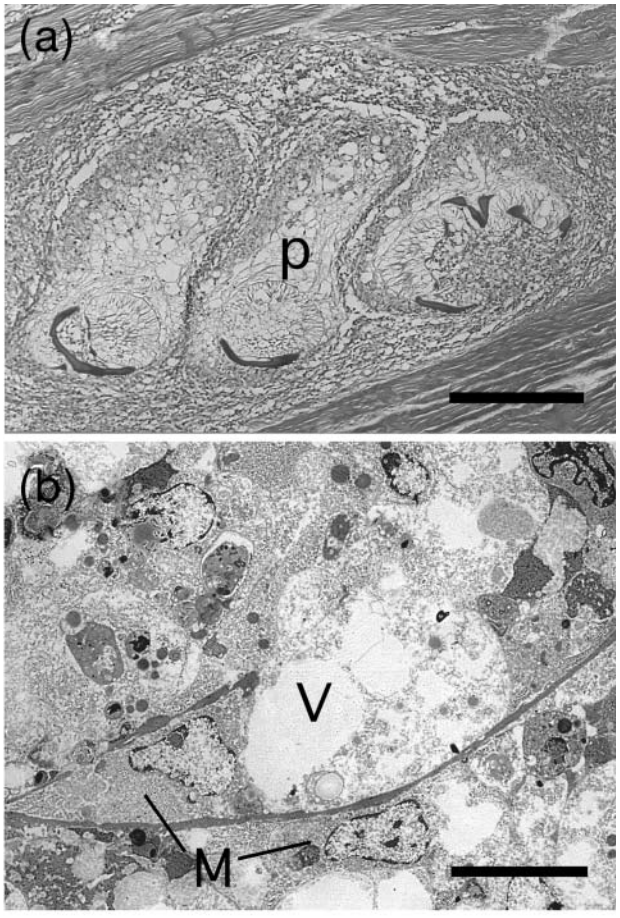

Fig. 5. *Neoheterobothrium hirame* infecting *Paralichthys olivaceus*. Light and electron micrographs of the infected site at 54 d post challenge. (a) The outline of the parasites (p) is no longer clearly visible (H&E stain). Scale bar = 200 µm. (b) Collapse of the inner tissue of a parasite. Some vacuolation (V) and macrophages (M) are observed in the inner tissue. Scale bar = 6 µm

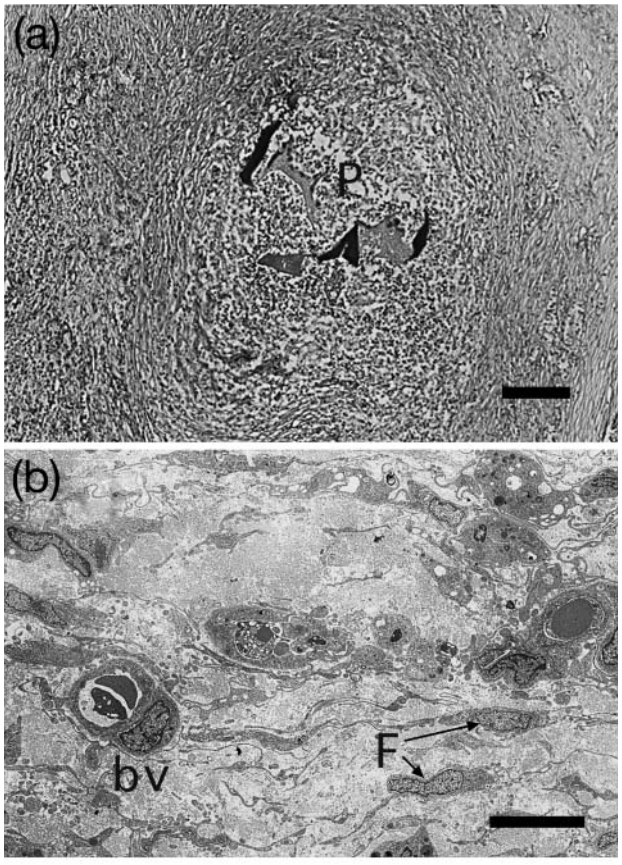

Fig. 6. *Neoheterobothrium hirame* infecting *Paralichthys olivaceus*. Light and electron micrographs of the buccal cavity wall after elimination of the parasite at 54 d post-challenge. (a) The attachment organ of the parasite (P) had almost disappeared and only remnants of the organ remain (H&E stain). Scale bar = 100 µm. (b) Generation of the blood vessels (bv) and migration of fibroblasts (F) are observed in the granulation tissue of the buccal cavity wall. Scale bar = 6 µm

DISCUSSION

The present results, together with the previous observations on the leucocyte types involved in the host response to *Neoheterobothrium hirame* (Nakayasu et al. 2003), show that infiltrating cells mainly consists of granulocytes, macrophages and DGCs. After the appearance of mature parasites, lymphocytes, thrombocytes and erythrocytes decreased rapidly in the blood as the anemia progressed. It is reported that the anemia caused by the parasites was characterized by heavy decrease of erythrocytes (Yoshinaga et al. 2001, Nakayasu et al. 2002a,b). This is considered to be caused by the blood feeding activity of the parasite. Therefore, lymphocytes and thrombocytes were probably consumed by the parasites along with erythrocytes. Nevertheless, granulocytes and monocytes increased in the blood, suggesting active recruitment of these cells. These cells infiltrated into the infected sites and adhered to the parasite's tegument. These results suggest that granulocytes and monocytes play important roles in the host response to the parasites. In addition, DGCs, which might be homologous to the eosinophilic granular cells (EGCs) of several fish species (Nakayasu et al. 2003) were sometimes observed in sites infected with parasites. In some fish species, the infiltration and adhesion of macrophages, neutrophils or EGCs against parasites have also been reported (e.g. Cross & Matthews 1993, Hoole & Nisan 1994, Huizinga & Nadakavukaren 1997) and are considered to be important steps in the elimination of parasites.

It seems that cells infiltrating infection sites attack the parasites directly, as suggested by the damage in

the parasite tegument to which the cells were attached on Day 47 post-infection. The release of cytotoxic factors by infiltrating cells seems to be especially important in antiparasitic responses. Studies on the *in vitro* killing of parasites by rainbow trout leucocytes suggest that reactive oxygen species (ROS) produced by the respiratory burst of macrophages evoked damage to the plerocercoid tegument of the cestode *Diphyllobothrium dendriticum* (Sharp et al. 1991) and killed the digenean, eye-fluke *Diplostomum spathaceum* (Whyte et al. 1989). Whyte et al. (1989) discussed the issue that the increased contact between macrophages and a parasite ensures the release of ROS and lytic enzymes, causing direct damage to the parasite. Hamers et al. (1992) suggested carp granulocytes caused damage to the metasoma tegument of *Paratenuisentis ambiguus* by the release of granule contents. The EGCs have also been reported to have a role in antiparasitic responses (Sharp et al. 1989, Hoole & Nisan 1994). Huizinga & Nadakavukaren (1997) suggested that the EGCs of goldfish *Carassius auratus* functioned as antiparasitic cells by releasing bioactive granules that altered the physiological environment of the infected sites and facilitated the expulsion of the trematode *Ribeiroia marini*. In the present study, the infiltrated cells also seemed to release cellular contents to the surface of the tegument of *Neoheterobothrium hirame*. The damage to the tegument may be caused by the release of cytotoxic factors from the infiltrated cells. Furthermore, many infiltrating macrophages and granulocytes had remnants of the tegument in their phagosomes on Day 47. These cells are known to be the principal phagocytic leucocytes in several fish species (e.g. Ellis 1977). Phagocytic cells play various roles, such as the recognition or elimination of invading pathogens or the scavenging of damaged cells or tissues. Cross & Matthews (1993) argued that phagocytic responses do not seem to cause direct damage to parasites, and that they may play a role in mediating a protective response. In the present study, these cells may also have scavenged debris of dead cells or led to the collapse of parasite tegument that was destroyed by the released chemicals.

Damage to the inner tissues of the parasite attachment organs seems to be caused by a large number of infiltrated cells in the later stage of reactions. Loss of tegument caused by the activities of the infiltrated cells will certainly allow invasion by the host cells and fluid into the inner tissues of the parasite. Cone et al. (1987) and Matthews & Matthews (1988) also suggested that the tegument serves as protection against both physicochemical conditions and mechanical damage by the host response. Furthermore, exposure of the inner tissues to the host humor/serum may exert an osmotic shock in addition to a toxic effect (Li & Arai 1988, Hamers et al. 1991).

The results of the present study suggest that monocytes/macrophages, granulocytes and DGCs play major roles in the non-specific immune response against *Neoheterobothrium hirame* during the infection period. However, the involvement of humoral factors such as immunoglobulin and complement cannot be discounted. In mammals, macrophages, neutrophils and eosinophils adhere directly to the schistosomula of the trematode *Schistosoma mansoni* via the Fc and C3b receptors and induce structural damage and death of this parasite (Butterworth 1984). In fish, immunoglobulin and complement have been implicated as opsonic factors in the adherence of leucocytes to parasites (Hoole & Arme 1986, 1988). Hence, further studies are needed to clarify whether humoral factors are involved in the host response to *N. hirame* in the Japanese flounder.

Elimination of the parasites was determined in the surviving fish belonging to the fed group. The results of the present study suggest that the elimination of *Neoheterobothrium hirame* was probably caused by the cellular response of the host fish. More specifically, the cellular response which caused the destruction of the parasite's posterior part containing the attachment organ was probably the major cause for the elimination of *N. hirame*. Elimination of the parasites was observed during almost the same period as that in which the destruction of the embedded part of the parasites was observed. On the other hand, since the length of time required for the elimination of parasites varied greatly among infected fish in this study, it seems unlikely that the aging of the parasites is a major factor in their detachment.

The starved challenged fish were not able to eliminate the parasites, and a high mortality was observed in this group. Since much more severe anemia was observed in this group, the death of these fish was probably caused by the anemia. Nakayasu et al. (2002b) reported that feeding was effective in preventing the progression of anemia induced by repeated bleeding from caudal blood vessels with syringes. In the present study, feeding was significant in preventing the development of anemia, resulting in a reduction of mortality in infected fish. This suggests that the fish can eliminate the parasites when they are fed sufficiently. In addition, it is well known that the nutritional condition greatly affects the immune function in mammals. In this study, although no difference was found in histological observations of the host response between the fed and starved groups, feeding might also have strengthened the immune activity itself, thus facilitating the elimination of the parasites.

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