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Immunolabelling of Fish Host Molecules on the Tegumental Surface of *Ligula Intestinalis* (Cestoda: Ppseudophyllidea)

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Abstract—Williams M. A. and Hoole D. 1995. Immunolabelling of fish host molecules on the tegumental surface of *Ligula intestinalis* (Cestoda: Pseudophyllidea). *International Journal for Parasitology* **25**: 249-256. Immunoblotting, SDS–PAGE and western blotting procedures were used to demonstrate cross-reactivity of a polyclonal anti-carp IgM antibody with components of roach serum. The polyclonal antibody labelled 2 major bands in both immune and normal roach sera corresponding to molecular masses of approximately 90 and 65 kDa. One of these bands (65 kDa) was considered to be the heavy chain of fish immunoglobulin whilst the identity of the other remains uncertain. This cross-reaction has been exploited in immunofluorescence and immunogold labelling studies to localize fish host molecules on the tegumental membrane of *Ligula intestinalis* freshly removed from roach fry. Immunogold studies revealed a low level of host molecules to be associated mainly with the microthrix spines of the tegument with less gold labelling being observed on the microthrix shafts.

Key words: Immunolabelling; *Ligula intestinalis*; tegumental antigens

INTRODUCTION

There have been several studies on the inflammatory response evoked in piscine hosts infected with pseudophyllidean tapeworms (Stromberg & Crites, 1974; Rosen & Dick, 1984; Otto & Heckmann, 1984; Sharp, Pike & Secombes, 1989). Arme & Owen (1968; 1970) noted that in the majority of cyprinids the plerocercoid of *Ligula intestinalis* (L.) elicited an intense cellular immune response which, in roach, *Rutilus rutilus* (L.), comprised macrophages, neutrophils, monocytes, vacuolated granulocytes and lymphocytes (Hoole & Arme, 1982; 1983a,b). Whether or not ligulosis elicits a humoral immune response has not been clarified. Molnár & Berczi (1965) showed that the serum from ligulosed bream,

Abranis brama (L.), contained a precipitin against *Ligula* which was demonstrable using a double diffusion assay. In contrast, Sweeting (1977), using the same technique, failed to demonstrate a worm-reactive precipitin in the serum of naturally ligulosed roach. However, in this latter study electrophoretic analysis did reveal an increase in the γ-globulin fraction of ligulosed roach serum relative to that of serum from non-ligulosed fish, a result possibly indicative of an antibody response. Williams & Hoole (1992) speculated that the apparent absence of antibody noted by Sweeting (1977) may have been due to the duration of infection and/or environmental temperature. Although our previous studies, using sera from naturally ligulosed roach, corroborated the work of Sweeting (1977) we also showed that when fish received an intraperitoneal injection

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of crude worm homogenates and were maintained at a temperature of 20°C a precipitin band could be obtained using the double diffusion technique.

In mammalian–helminth symbioses both antibody and complement can act as mediators in the immune interactions between leucocytes, humoral factors and helminths (Butterworth, 1984; Wakelin, 1986). The role of serum components in the fish host response to ligulosis has been investigated by Hoole & Arme (1986) using an in vitro cell adherence assay in which worms were exposed to fish leucocytes in the presence of various serum components. Both antibody and a complement protein, probably C3, were implicated as ligands in the mediation of cell attachment. In addition, the results of similar assays carried out by Hoole & Arme (1988), in which sera was treated with phosphorylcholine (PC), suggested that the acute-phase protein, C-reactive protein (CRP), possibly in association with complement, may also be involved in leucocyte adherence to the metacestode.

Studies on other fish–helminth symbioses have also implicated antibody ligands in the mediation of cellular attack. For example, Bortz, Kenny, Pauley, Garcia-Ortigoza & Anderson (1984) have reported antibodies in the sera of trout, Oncorhynchus mykiss, infected with the eye fluke Diplostomum spathaceum, whilst investigations by Whyte, Chappel & Secombes (1989) have suggested an antibody-dependent cell-mediated cytotoxicity mechanism in the immune response of this fish species to diplostomula of D. spathaceum. Of particular interest are the in vitro investigations by Sharp et al. (1989) in which worm-specific antibodies were demonstrated on the surface of isolated plerocercoids of Diphyllobothrium dendriticum and D. ditremum exposed to immune serum. However, antibodies were not detected on the surface of worms in situ using immunofluorescence on cryostat sections of intact cysts.

Recent investigations on the roach–Ligula model by Williams & Hoole (1992) revealed that intraperitoneal injections of worm homogenates elicited an increase in the percentage number of antibody-producing cells in the pronephros and spleen of experimental fish. The fate of secreted antibodies was unknown but in natural infections a role in opsonization of the parasite, either to mediate cellular attachment or to initiate complement activation, has been proposed (Hoole & Arme, 1986). Clearly, the direct demonstration of host antibody on the surface of uncultured Ligula would be of value in elucidating the role of the humoral immune response to this parasite.

Initial experiments have been carried out to establish whether a polyclonal anti-carp IgM antibody could react with roach antibody. Once this had been established the polyclonal antibody was then used in immunofluorescence and immunogold labelling assays to localize putative fish antibodies on the surface of Ligula intestinalis.

MATERIALS AND METHODS

Sera. Six non-ligulosed roach from Highfield Pool, Nottinghamshire, U.K. (mean fork length 14.6 ± 0.1 cm) were maintained at 12°C ± 1°C in dechlorinated, aerated tapwater. After anaesthetising in MS222 each fish received a 0.2 ml intraperitoneal (IP) injection of Ligula tegumental homogenates (protein concentration 0.3 mg/ml) in Freunds Complete Adjuvant (FCA) (1:1) on day 0 and again on day 14. Tegumental homogenates were obtained by freeze-thawing of whole worms, washing in 0.85% saline and removing the supernatant. Fish were bled, under anaesthetic, by caudal puncture one week after the second injection, i.e., day 21 post-primary injection, and blood allowed to clot for 1.5 h at 4°C, after which the serum (immune roach serum; IRS) was removed, pooled and frozen at --20°C until required. Pooled normal roach serum (NRS) was obtained from 6 non-ligulosed fish, mean fork length 13.2 ± 0.1 cm. Immune carp serum (ICS) was obtained from carp, *Cyprinus carpio*, fork length 10–15 cm, infected with the tapeworm *Bothriocephalus achielognathi*. Anti-carp IgM polyclonal antibody (α-cIgM), raised in Dutch white rabbits, was kindly supplied by Drs M. Cross and R.M. Matthews of Plymouth University, U.K. Peroxidase-labelled goat anti-rabbit IgG (peroxidase α-rIgG) was obtained from Sigma Chem. Co.

Immunoblotting. In each assay antibodies were diluted in blocking solution (PBS, 1% dried milk powder, 0.01% Tween 20). Five rows, each comprising 4 spots of 5 µl reagent, were immobilized on to 4 sheets of nitrocellulose blotting paper (Sartorius). The first row, comprising IRS, was spotted in dilutions of 1:1, 1:2, 1:4 and 1:8. Similar dilutions of sera were used in the controls, i.e. a negative control of normal roach serum in row 2 and a positive control of ICS in row 3. Row 4 contained spots of blocking solution whilst the fifth row, which served as a positive colour control, comprised α-cIgM polyclonal antibody at a dilution of 1:200. After incubating at room temperature (approximately 20°C) the nitrocellulose papers were immersed for 30 min in blocking solution and washed 3 × 5 min in PBS, 0.01% Tween 20. Primary antibody (α-cIgM) was added at dilutions of either 1:50, 1:100, 1:200 or 1:400, each dilution being placed on to one of 4 sheets. The primary antibody was then left to incubate overnight at room temperature. After removal of the primary antibody and washing, the nitrocellulose papers were incubated in a 1:1000 dilution of peroxidase-conjugated α-rIgG for 15–30 mins. After washing, the sheets were developed for 10 min in a solution of 0.6mg/ml chloronapthol/0.05% hydrogen peroxide in PBS, and then rinsed in distilled water.

Western blotting. NRS and IRS were diluated 1:4 in sample buffer (0.2 M-Tris base, 1.0 M-sucrose, 5% mercaptoethanol, 0.01% Bromophenol blue, pH 8.8) and 10 µl of each solution placed into a well in a 10% SDS–PAGE gel.

Ten μl of molecular mass markers comprising 180, 116, 84, 50 and 48 kDa protein subunits (Sigma) were used as comparisons. Electrophoresis was carried out using an LKB "Mighty Small" PAGE electrophoresis unit connected to an LKB Bromma 2197 40 mA power supply. Following separation proteins were electrophorectically transferred from the gels on to nitrocellulose blotting paper using a TE70 Semi-dry transfer unit (Hoefer Scientific Instruments) run at 80 mA. Nitrocellulose papers were temporarily stained with 0.2% Ponceau Red for 5 min to check that all bands had been transferred. The papers were then destained in distilled water and the proteins probed with a 1:200 dilution of rabbit α-cIgM. Following this a 1:1000 dilution of peroxidase conjugated goat α-rIgG was added for 15–30 min. Final visualization was carried out as described above.

Immunofluorescence labelling. Ligulosed roach fry, mean weight 0.65 ± 0.01 g, were obtained from Tittesworth Reservoir, Staffordshire, U.K. Fish were maintained in dechlorinated, aerated tapwater at 12 ± 1°C and fed on commercial fish meal. Worms were removed under teleost Ringer (Lockwood, 1963) using parafilm-covered forceps and washed 3 × in fresh Ringer solution to remove any adherent host cells. The worms were then cut into approximately 3 mm pieces and each section prefixed in 2% paraformaldehyde solution in PBS (pH 7.4) for 4–6 h. After prefixing, worms were washed first in teleost Ringer and then PBS–1%BSA. After blocking in 3% BSA in PBS for 2 h, the tissue was again washed in PBS–1%BSA, and immersed for 2 h in primary antibody (α-cIgM) in PBS at dilutions of 1:20, 1:50 and 1:100. Worm material was then washed and blocked as above and then incubated for 2 h in the secondary antibody, FITC conjugated α-rIgG, at a dilution of either 1:10, 1:20, 1:40 or 1:80 in PBS. After washing, specimens were viewed using a Reichert Zetopan microscope with a twin lamp Binolux II source, set up for epifluorescence. Photographs were taken using Kodak EKTAR 1000/31° film. In addition to the above, four controls were prepared: (a) to check for autofluorescence, worms were incubated in PBS-3%BSA, in the absence of antibodies; (b) to check for non-specific binding of the secondary antibody (FITC α-rIgG) the α-cIgM stage was omitted, (c) to check for non-specific binding of components other than antibody in the immune rabbit serum, normal rabbit serum was included at a dilution of 1:50, before applying the secondary antibody; (d) IRS was included before the application of the secondary antibody. This addition would check for and eliminate unbound α-IgM in the assay.

Immunogold labelling. Worm material, washed and blocked as described above, was incubated for 2 h in primary antibody (polyclonal α-cIgM) in 0.5 MNaCl, 0.1%BSA, 0.05% Tween 20, 5% Foetal Bovine Serum at a dilution of either 1:10, 1:20, 1:50 or 1:100. After washing, the material was immersed for 2 h in the secondary antibody, goat anti-rabit IgG-gold conjugate (α-rIgG gold), 10 nm particle size (Sigma) in 0.05 MNaCl, 0.01%BSA, 0.05% Tween 20, 5% Foetal Bovine Serum at dilutions of 1:10, 1:20, 1:40 or 1:80, and postfixed overnight in 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.2)

containing 2 mM-CaCl. The material was washed for 4 h in 0.1 M-sodium cacodylate, 2 mM-CaCl and finally post-fixed in 1% osmium tetroxide in cacodylate buffer for 2 h. After dehydration in a graded series of acetone (30, 60, 90, 100%) parasite material was embedded in Spurr's resin. Ultra-thin sections were viewed unstained using a Jeol 100 CX II electron microscope at 80 kV. Control preparations were as for immunofluorescence with the exception of the check for autofluorescence.

RESULTS

Immunoblotting

The results of the immunoblotting procedure are shown in Fig. 1. Row 3 (ICS) clearly shows binding of the α-cIgM antibody to whole immune carp serum at all dilutions. IRS (Row 1) also bound the α-cIgM antibody with slightly less colour being developed in the neat, 1:2 and 1:4 dilutions than in the corresponding dilutions of ICS. At a dilution of 1:8 the amount of antibody binding appeared similar in both IRS and ICS. NRS (Row 2) also showed some reactivity with the α-cIgM antibody. This was expected since it is highly probable that NRS would contain unknown titres of antibody. However, the reaction product was less intense with NRS than with either of the immune sera suggesting that the antibodies in NRS may be present at lower levels.

Western blotting

The original polyacrylamide gel, after electrophoresis of proteins, is shown in Fig. 2. Many of the protein bands, particularly 100, 90 and 65 kDa (Fig. 2 arrows) appeared more dense in IRS than those in NRS, perhaps suggesting that immunization results in increased levels of these serum proteins. western blotting (Fig. 3) revealed binding of the α-cIgM antibody to be specific for two major bands, corresponding to 90 and 65 kDa, in both NRS and IRS.

Immunofluorescence labelling

Dilutions of 1:50 α-cIgM and 1:20 FITC-conjugated α-rIgG were found to give optimal binding. Labelling worm sections with FITC-labelled antibodies resulted in fluorescence, probably due to a greater depth of field, being observed at the periphery of the worm (Fig. 4). Although not clearly visible on Fig. 4, the surface of the worm also displayed faint fluorescence. Fluorescence was not observed in any of the control experiments with the exception of (d), where IRS was added to sequester any unbound α-cIgM. In this control the level of fluorescence was indistinguishable from experimental specimens.
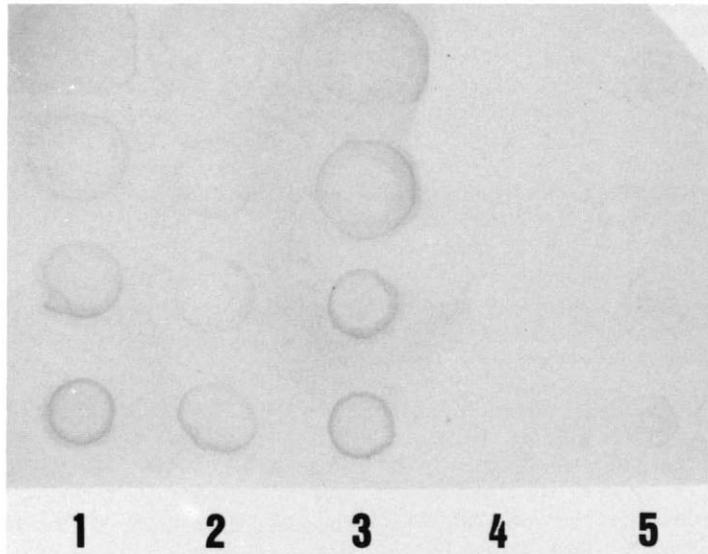
Fig. 1. Immunoblotting of polyclonal α-cIgM (1:200) onto IRS (1), NRS (2) and
ICS (3) at 4 different dilutions (1:1, 1:2, 1:4, 1:8 top to bottom). Rows 4 and
5 represent blocking solution and a positive colour control, respectively.

Immunogold labelling

Dilutions of 1:50 polyclonal α-cIgM and 1:40 gold conjugate antibodies were found to produce optimal binding. Gold labelling of the apical plasma membrane/glycocalyx was relatively sparse with particles observed attached to the microtriches particularly associated with the microthrix spines (Fig. 5). Only rarely were gold-conjugated antibodies
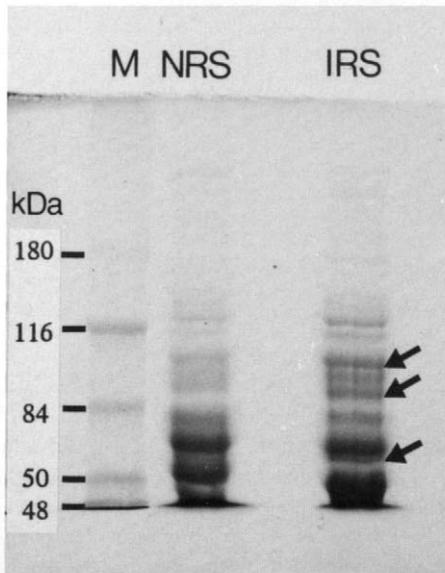

Fig. 2. SDS–PAGE of NRS and IRS revealed at least 3 bands, corresponding to molecular masses of approximately 100, 90 and 65 kDa (arrows), to be more pronounced in IRS than in NRS. Molecular mass markers (M) are shown on the left.
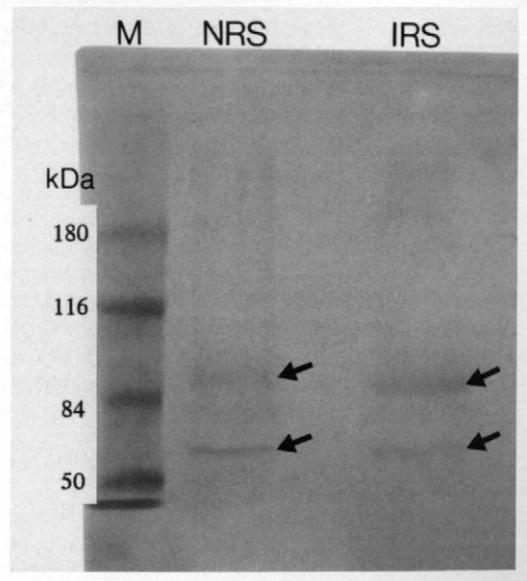

Fig. 3. Western blotting of the SDS–PAGE gel shown in Fig. 2 displayed 2 bands, corresponding to molecular masses of approximately 90 kDa and 65 kDa (arrows), to be specifically labelled with the α-cIgM antisera in both NRS and IRS Molecular mass markers (M) are shown on the left.
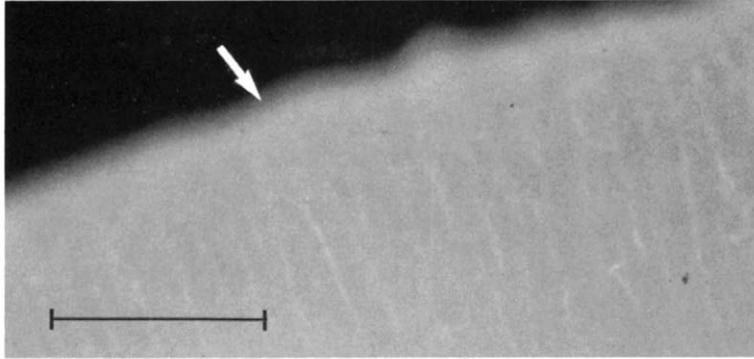
Fig. 4. Immunofluorescence labelling of fish host molecules on the worm surface using FITC-labelled α-rIgG and α-cIgM antisera. Fluorescence was clearly visible at the periphery of the worm (arrow). Scale bar = 1 mm.

observed attached to the membrane/glycocalyx of the proximal microthrix border. As with the fluorescence experiments the only control to display gold labelling was (d), which incorporated IRS to sequester α-cIgM not bound to the parasite surface.

DISCUSSION

The tegumental surface of helminths has been the subject of much research (e.g. Oaks & Lumsden, 1971; Befus, 1977; Friedman, Weinstein, Davidson & Mueller, 1982; Schmidt & Peters, 1987; Lightowlers & Rickard, 1988).
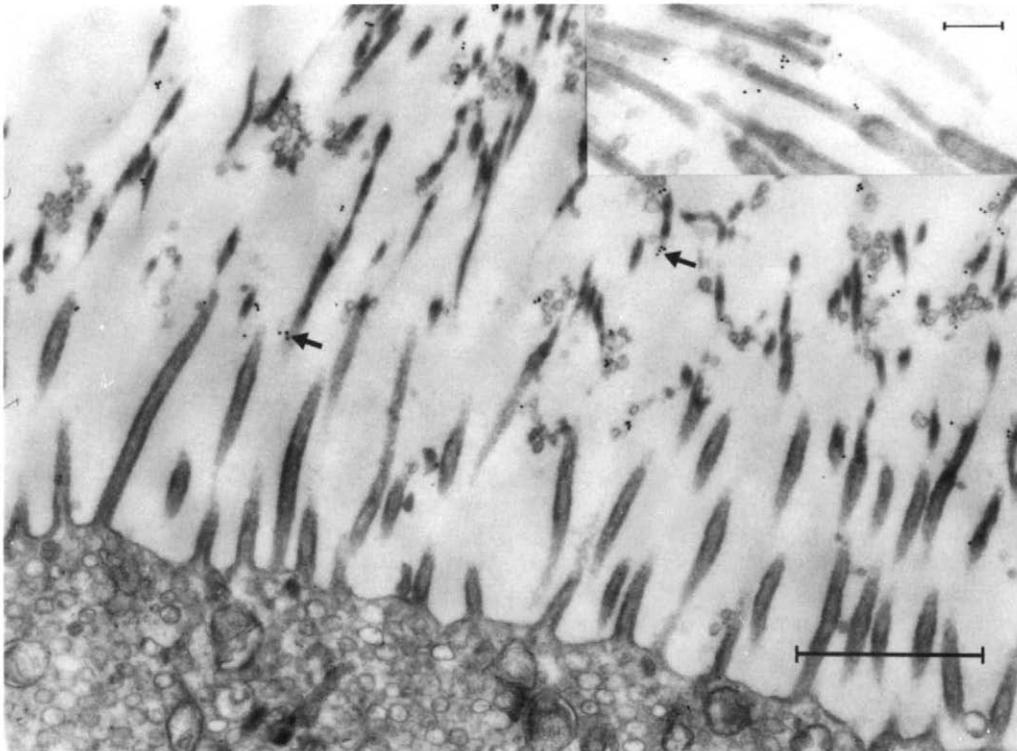

Fig. 5. Immunogold labelling of fish host molecules on the apical plasma membrane of *L. intestinalis* using gold-labelled α-rIgG and α-cIgM antisera. Gold particles were mainly observed bound to the distal microthrix border (arrows) and associated with the microthrix spines (inset) Scale bars = 1μm (main plate) and 0.2 μm (inset).

The surface of the plerocercoid of *Ligula intestinalis*, as revealed by the electron microscope, resembles that of other cestodes and is assumed to be polyionic (Hoole & Arme, 1985). The microthrix border is, however, unusually thick and can extend to a depth of 20 µm, with each microthrix being composed of a relatively short shaft and long, flexible spine. The presumed polyionic nature of the surface would suggest that host proteins, including antibody and complement molecules, may be adsorbed on to the surface membrane of worms *in situ* (Hoole & Arme, 1985).

Experiments utilizing SDS–PAGE and western blotting procedures have demonstrated that a polyclonal antibody raised against carp immunoglobulin (Ig) cross-reacts with components in xenogenic roach serum. Two bands, corresponding to molecular masses of approximately 90 and 65 kDa, were specifically recognized by the polyclonal. These bands fall within the range of the molecular masses of the heavy chain component of antibodies from other cyprinids, i.e. goldfish, carp and tench, reported by Vilain & Wetzel (1984). In this latter study Ig light chains had weights of approximately 25 kDa that remained relatively consistent between species.

Since the molecular masses of the bands observed in our experients is well in excess of that observed for the light chains of Ig found in other fish species, it is suggested that one of these bands, probably the 65 kDa band, represents the heavy chain of roach Ig. The nature of the second band remains unknown but its molecular mass is reasonably consistent with that found for the lighter subunits of some components of the complement system. For example, rainbow trout C3 is composed of two polypeptide chains of molecular masses 128 and 74 kDa (Nonaka, Iwaki, Nakai, Nozaki, Kaidoh, Nonaka, Natsuume-Sakai & Takahashi, 1984)·whilst rainbow trout C5 subunits have molecular masses of 133 kDa and 86 kDa (Nonaka, Natsuume-Sakai & Takahashi, 1981).

Immunolabelling of the tegument of *L. intestinalis* has demonstrated a distribution of host molecules to be associated mainly with the microthrix spines, with less binding being observed proximally. Whether one or both of the serum components shown to be specifically bound by the anti-carp polyclonal are being labelled on the worm surface cannot be known from our experiments. However, the distal distribution of host molecules presumably reflects a differential distribution of parasite antigens. Such a localized distribution of parasite antigens has also been reported by other workers. Befus (1977) used an

immunofluorescence technique to demonstrate host antibodies on the surfaces of *H. diminuta* and *H. microstoma* from mice. The distribution of fluorescence on the surface of these worms was different according to the antisera used. IgA was shown to be located linearly at the worm vela whereas the C3 complement component displayed a granular fluorescence. In addition, Schmidt & Peters (1987), using gold-labelled lectins, visualized glycoconjugates on the microthrix spines of the tegumental surfaces of *H. diminuta* and *H. microstoma*. Less binding was observed over the proximal parts of the microtriches.

The precise nature of the parasite antigens being recognized is not known. However, phosphorylcholine (PC), although never having been directly demonstrated to be a component of the *Ligula* tegumental membrane is a ubiquitous molecule that has been found to be a component of other helminths (Péry, Petit, Poulain & Luffau, 1974; Gutman & Mitchell, 1977; Fletcher, White & Baldo, 1980). Indirect evidence for a PC component of the *Ligula* membrane comes from the cell adherence assays, using PC-treated sera, of Hoole & Arme (1988). These experiments implicated a possible role for CRP in mediating the cellular attack of roach to the parasite. In addition, Hoole & Arme did not discount the possibility that anti-PC antibodies were involved.

Alternatively the parasite molecules may be ES products of metabolism. Lightowlers & Rickard (1988) reviewed the role of ES products of helminths on the host immune system. Worm ES products may evoke an effective immune response or, alternatively, they may provide the parasite with a means by which immune evasion can be secured (Lightowlers & Rickard, 1988). A dramatic example of the latter is the excretion of a polysaccharide by *Taenia taniaeformis* that is able to consume complement at some distance away from the parasite surface (Hammerberg & Williams, 1978).

Such sequestration of immune components has also been suggested for a fish cestode by the investigations of Sharp *et al.* (1989). Utilizing an *in vitro* immunofluorescence technique they demonstrated the presence of specific antibodies on the tegument of two species of *Diphyllobothrium* from trout when the worms were incubated in pooled immune serum. However, *in situ* experiments using cryostat sections through intact cysts did not reveal the presence of fish antibodies associated with the worm tegumental surface. Of particular interest were the high levels of fish antibody demonstrated on the inner margins of cysts. Since this site was shown to be composed of detached microtriches in fibrous tissue it was

suggested that the passive absorption of antibody in this area may aid in parasite survival. In the roach–*Ligula* model a layer of host cellular remnants and parasite microthrix fragments has been observed adjacent to worms obtained from roach fry (Hoole & Arme, 1983a). If a similar mechanism of host antibody absorption, as that suggested for *Diphyllobthrium* spp. (see Sharp *et al.*, 1989) occurs in the roach–*Ligula* model, this may account for the paucity of host molecules demonstrated on the tegumental surface of *L. intestinalis*.

It is a characteristic of ligulosis that, regardless of the extensive cellular onslaught seen in the majority of fish hosts, the worm can survive the life of its host. Indeed, in the gudgeon, *Gobio gobio*, not only does *Ligula* survive the duration of the life of its host but, by a mechanism not yet understood, the worm also avoids eliciting a host cellular immune response (Taylor & Hoole, 1989). In roach, *in vivo* tegumental damage to *Ligula* is restricted to phagocytosis of the microtriches by macrophages (Hoole & Arme, 1982) with extensive damage to the parasite being limited possibly due to the physical inability of the fish leucocytes to penetrate the dense microthrix border. Alternatively, serum components, such as antibody or complement, which are capable of opsonization of the worm tegument, may bind to antigens which are themselves restricted in their distribution on the tegumental surface, as would be suggested from the results described in the present paper.

In conclusion, the localization of host molecules over the distal microthrix of *L. intestinalis* suggests a localization of antigens similar to that observed in other cestodes. In addition, the paucity of immune labelling may be the result of a sequestration zone around the parasite, a hypothesis that requires further investigation. The distribution of host molecules that could possibly act as ligands is consistant with the findings of Hoole & Arme (1982; 1983a,b) who infrequently observed leucocyte penetration of the microthrix border. They suggested that the lack of cellular penetration was a physical phenomenon. Based on our observations it is proposed that the distal localization of the cellular response may be a result, at least in part, of the restricted distribution of host ligands and their corresponding parasite immunogens.

REFERENCES

Arme C. & Owen R. W. 1968. Occurrence and pathology of *Ligula intestinalis* infections in British Fishes. *Journal of Parasitology* **54**: 272–280.

Arme C. & Owen R. W. 1970. Observations on a tissue response within the body cavity of fish infected with the plerocercoid larvae of *Ligula intestinalis* (Cestoda: Pseudophyllidea). *Journal of Fish Biology* **2**: 35–37.

Befus A. D. 1977. *Hymenolepis diminuta* and *H. microstoma*: Mouse immunoglobulins binding to the tegumental surface. *Experimental Parasitology* **41**: 242–251.

Bortz B. M., Kenny G. E., Pauley G. B., Garcia-Ortigoza E. & Anderson D. P. 1984. The immune response of immunized and naturally infected rainbow trout (*Oncorhynchus mykiss*) to *Diplostomum spathaceum* as detected by enzyme-linked immunosorbent assay (ELISA). *Developmental and Comparative Immunology* **8**: 813–822.

Butterworth A. E. 1984. Cell-mediated damage to helminths. In: *Advances in Parasitology*, Vol. 23 (edited by Baker J. R. and Muller R.), pp. 144–235. Academic Press, London.

Fletcher T. C., White A. & Baldo B. A. 1980. Isolation of a phosphorylcholine-containing compound from the turbot tapeworm, *Bothriocephalus scorpii* (Müller), and its reaction with C-reactive protein. *Parasite Immunology* **2**: 237–248.

Friedman, P. A., Weinstein P. P., Davidson L.A. & Mueller J. F. 1982. *Spirometra mansonoides*: lectin analysis of tegumental glycopeptides. *Experimental Parasitology* **54**: 93–103.

Gutman G. A. & Mitchell G. F. 1977 *Ascaris suum*: location of phosphorylcholine in lung larvae. *Experimental Parasitology* **43**: 161–168.

Hammerberg B. & Williams J. F. 1978. Interaction between *Taenia taniaeformis* and the complement system. *Journal of Immunology* **120**: 1033–1038.

Hoole D. & Arme C. 1982. Ultrastructural studies on the cellular response of roach, *Rutilus rutilus* L., to the plerocercoid larva of the pseudophyllidean cestode, *Ligula intestinalis*. *Journal of Fish Diseases* **5**: 131–144.

Hoole D. & Arme C. 1983a. *Ligula intestinalis* (Cestoda: Pseudophyllidea): an ultrastructural study on the cellular response of roach fry, *Rutilus rutilus*. *International Journal for Parasitology* **13**: 359–363.

Hoole D. & Arme C. 1983b. Ultrastructural studies on the cellular response of fish hosts following experimental infection with the plerocercoid of *Ligula intestinalis* (Cestoda: Pseudophyllidea). *Parasitology* **87**: 139–149.

Hoole D. & Arme C. 1985. The *in vitro* culture and tegumental dynamics of the plercercoid of *Ligula intestinalis* (Cestoda: Pseudophyllidea). *International Journal for Parasitology* **15**: (6) 609–615.

Hoole D. & Arme C. 1986. The role of serum in leucocyte adherence to the plerocercoid of *Ligula intestinalis* (Cestoda: Pseudophyllidea). *Parasitology* **92**: 413–424.

Hoole D. & Arme C. 1988. *Ligula intestinalis* (Cestoda: Pseudophyllidea): phosphorylcholine inhibition of fish leucocyte adherence. *Diseases of Aquatic Organisms* **5**: 29–33.

Lightowlers M. W. & Rickard M. D. 1988. Excretory–secretory products of helminth parasites: effects on host immune responses. *Parasitology* **96**: S123–S116.

Lockwood A. P. M. 1963. *Animal body fluids and their regulation.* Weinemann, London.

Molnar Von K. & Berczi I. 1965. [Demonstration of parasite-specific antibodies in fish blood by agar gel diffusion precipitation test]. *Zeitschrift für Immunitäts Allergie-Forschung* **129**: 263–267.

Nonaka, M., Natsuume-Sakai S. & Takahashi M. 1981. The complement system in rainbow trout (*Oncorhynchus mykiss*) II. Purification and characterization of the fifth component (C5). *Journal of immunology* **126**: 1495–1498.

Nonaka, M., Iwaki M., Nakai C., Nozaki M., Kaidoh T., Nonaka M., Natsuume-Sakai S. & Takahashi M. 1984. Purification of a major serum protein of rainbow trout (*Oncorhynchus mykiss*) homologous to the third component of mammalian complement. *Journal of Biological Chemistry* **259**: 6327–6333.

Oaks J. A. & Lumsden R. D. 1971. Cytological studies on the absorptive surfaces of cestodes. V. Incorporation of carbohydrate-containing macromolecules into tegument membranes. *Journal of Parasitology* **57**: 1256–1268.

Otto T. N. & Heckmann R. A. 1984. Host tissue response for trout infected with *Diphyllobothrium cordiceps* larvae. *Great Basin Naturalist* **44**: 125–132.

Pery P., Petit A., Poulain J. & Luffau G. 1974. Phosphorylcholine-bearing components in homogenates of nematodes. *European Journal of Immunology* **4**: 637–639.

Rosen R. & Dick T. A. 1984. Experimental infections of rainbow trout, *Salmo gairdneri* Richardson, with the plerocercoids of *Triaenophorus crassus* Forel. *Journal of Wildlife Diseases* **20**: 34–38.

Schmidt J & Peters W. 1987. Localization of glycoconju-gates at the tegument of the tapeworms *Hymenolepis nana* and *H. microstoma* with gold-labelled lectins. *Parasitology Research* **73**: 80–86.

Sharp G. J. E., Pike A. W. & Secombes C. J. 1989. The immune response of wild rainbow trout, *Salmo gairdneri* Richardson, to naturally acquired plerocercoid infections of *Diphyllobothrium dendriticum* (Nitzsch, 1824) and *D. ditremum* (Creplin, 1825). *Journal of Fish Biology* **35**: 781–794.

Stromberg P. C. & Crites J. L. 1974. Triaenophoriasis in Lake Erie white bass, *Morone chrysops. Journal of Wildlife Diseases* **10**: 352–358.

Sweeting R. A. 1977. Studies on *Ligula intestinalis*: some aspects of the pathology in the second intermediate host. *Journal of Fish Biology* **10**: 43–50.

Taylor M. & Hoole D. 1989. *Ligula intestinalis* (L.) (Cestoda: Pseudophyllidea): plerocercoid-induced changes in the spleen and pronephros of roach, *Rutilus rutilus* (L.), and gudgeon, *Gobio gobio* (L.) *Journal of Fish Biology* **34**: 583–596.

Vilain C. & Wetzel M. C. 1984. Structural and functional analysis of spontaneous anti-nitrophenyl antibodies in three cyprinid fish species: carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tench (*Tinca tinca*). *Developmental and Comparative Immunology* **8**: 611–622.

Wakelin D. 1986. Immunity to helminths. *EOS — Revista di Immunologia ed Immunofarmacologia* **6**: 35–46.

Whyte S. K., Chappell L. H. & Secombes C. J. 1989. Cytotoxic reactions of rainbow trout, *Salmo gairdneri* Richardson, macrophages for larvae of the eye fluke *Diplostomum spathaceum* (Digenea). *Journal of Fish Biology* **35**: 333–345.

Williams M.A. & Hoole D. 1992. Antibody response of the fish *Rutilus rutilus* to the metacestode of *Ligula intestinalis. Diseases of Aquatic Organisms* **12**: 83–89.