In vitro interactions between epithelial cells and *Gyrodactylus derjavini*

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Abstract

Skin responses of fish to various parasites have been shown to involve various immunologically competent cells producing factors which guide the reactions of epithelial cells. However, the present study has demonstrated that a monoculture of epithelial cells has the ability to encapsulate and partially degrade ectoparasites without involvement of leukocytes. The ectoparasitic monogenean *Gyrodactylus derjavini* was kept on a monolayer of Epithelioma Papulosum Cyprini (EPC) cells in 24-well multidishes supplied with tissue culture medium. *Gyrodactylus derjavini* did not reproduce but survived an incubation period of up to139 h in the system. Due to sterile conditions, dead gyrodactylids were not subjected to microbial degradation and remained intact for several weeks. However, at 40 days *G. derjavini* was overgrown by EPC-cells and became partly degraded during the following 15 days. Analysis of enzyme reactivity in EPC-cells showed reactions for ten enzymes including esterases, amidases, phosphatases and phosphohydrolases. No marked differences for the ten enzymes between cell cultures with and without the ectoparasites were found but it cannot be excluded that some of these enzymes took part in parasite degradation. The study showed the *in vitro* capability of epithelial cells to interact, encapsulate and degrade *G. derjavini* without the involvement of leukocytes. This response probably is non-specific and will not exclude that various immunocompetent cells and their products normally optimize and accelerate elimination of invading parasites *in vivo*.

Introduction

Several studies have described epithelial reactions in fish towards ectoparasitic monogeneans. Decades ago it was shown that *Dactylogyrus vastator* and *D. anchoratus* on the gills of carp (Wunder, 1929; Prost, 1963) and *D. macracanthus* on tench gills (Wilde, 1935) caused extensive cellular reactions in gill tissue. Epithelial outgrowths partly embedding the monogenean in gill tissue were also seen in blue ling infected with *Linguadactyla molvae* (Bychowsky, 1957), *Amphibdella flavolineata* on rays (Llewellyn, 1960), *Diplectanum aequans* on sea bass (Oliver, 1977), *Cleidodiscus robustus* on bluegill (Thune & Rogers, 1981), *Callorhynchicola multitesticulatus* on plownose chimaeras (Llewellyn & Simmons, 1984), *Lamellodiscus major* on sparids (Roubal, 1986) and *Pseudodactylogyrus bini* on eels (Buchmann, 1988). Various cell types were suggested to take part in the gill tissue reactions of carp against *D. vastator* (Paperna, 1964) and grass-carp against *D. lamellatus* (Molnar, 1972) and it is generally assumed that intricate immunological reactions are associated with these epithelial reactions (Buchmann, 1999). The roles of the different cell types in these host responses can be elucidated by studies on isolated cells and their interactions with the monogenean in question. Host macrophages and their effects on *Gyrodactylus derjavini* were studied by Buchmann & Bresciani (1999) and the present work describes the capability of isolated epithelial cells to encapsulate this monogenean.

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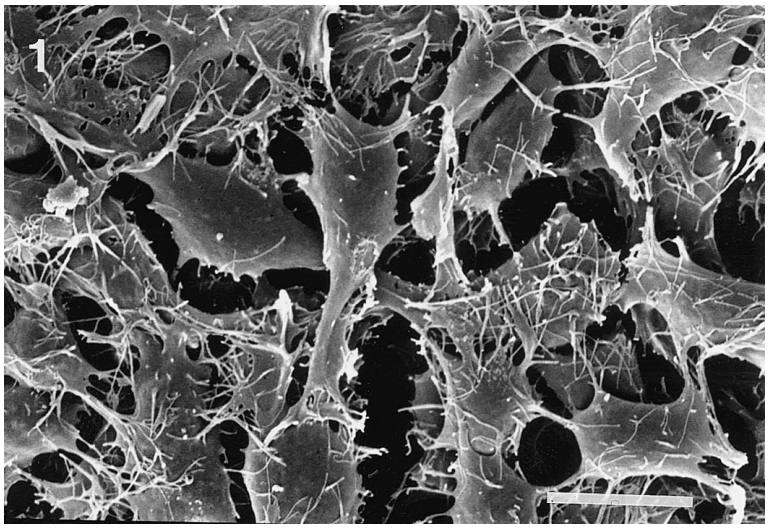

Fig. 1. EPC-cells in a loose monolayer 4 days after seeding in 24-well Nunc multidishes. SEM. Scale bar 10 μm.

Materials and methods

Parasites

Parasites (*Gyrodactylus derjavini*) were from a 3-year-old laboratory culture originally isolated from a Danish rainbow trout farm. They were kept on rainbow trout in 200 l aquaria with aerated freshwater (pH 7.4) (with a continous supply of uninfected fish) in a temperature controlled room (11–12°C) artificially illuminated on a 12 h light and 12 h dark cycle. Fish were killed with a blow to the head and fins were cut and placed in petri dishes with water. Living detached parasites were collected from the petri dish by the use of a needle to which the gyrodactylids attached. They were then transferred to wells in multidishes (Nunc, Denmark) as described below.

Cell culture

Monolayers of Epithelioma Papulosum Cyprini (EPC) cells (Fijan *et al.*, 1983) were prepared in 24-well multidishes (Nunc, Denmark) in tissue culture medium E-MEM (Sigma) and incubated at 11.6°C. The E-MEM was supplemented with 5% foetal calf serum (FCS), 5% heat inactivated fresh rainbow trout serum, penicillin (100 IU ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). Seeded cells were allowed to develop monolayers for 2–3 days before experimental use.

Experimental design

Two experiments were conducted (table 1). Parasites (1–3 $well^{-1}$) were incubated (at 11–12°C) in freshwater (seven wells), in E-MEM without cells but supplemented with 5% FCS and 5% rainbow trout serum (nine wells) and in EPC-cells supplied with FCS and fish serum as described above (24 wells). All wells contained penicillin and streptomycin to secure sterile conditions. Parasite survival was recorded daily for 7 days and then cell cultures were inspected weekly with an inverse microscope (Olympus CK40-F200) (magnification 40–400×). After 4, 40 and 55 days monolayers were prepared for scanning electron microscopy (SEM).

Enzymes

Monolayers of EPC-cells in three wells with or without parasites were rinsed with a solution of penicillin (500 IU ml^{-1}) and streptomycin (500 $\mu g\,ml^{-1}$) and finally suspended with a pipette in 3 ml of this solution. These cell suspensions were then added (65 μl $well^{-1}$) to 20 wells (one control and 19 tests) in a commercial enzyme test system (APIZYM, Biomerieux, France). After 4 h incubation at 20°C the enzyme reactivity was recorded from colour development according to the manufacturer's instructions.

Scanning electron microscopy

Monolayers of EPC-cells (after 4 and 40 days incubation), EPC-cells totally or partly embedding specimens of *G. derjavini* (after 40 and 55 days incubation) and parasite specimens incubated in tissue culture medium (40 days) were rinsed with phosphate buffered saline (PBS, pH 7.1) and fixed with cacodylate buffered glutaraldehyde (2.5%). After post-fixing in osmium tetroxide and critical

Table 1. Survival of *Gyrodactylus derjavini* in 24-well multidishes with water, E-MEM and EPC-cell cultures (1–3 parasites $well^{-1}$).

Incubation medium:	Water	E-MEM	EPC
Experiment I			
No. of parasites	13	13	35
Max. survival time	46 h	96 h	96 h
Experiment II			
No. of parasites	4	7	22
Max. survival time	26 h	118 h	139 h

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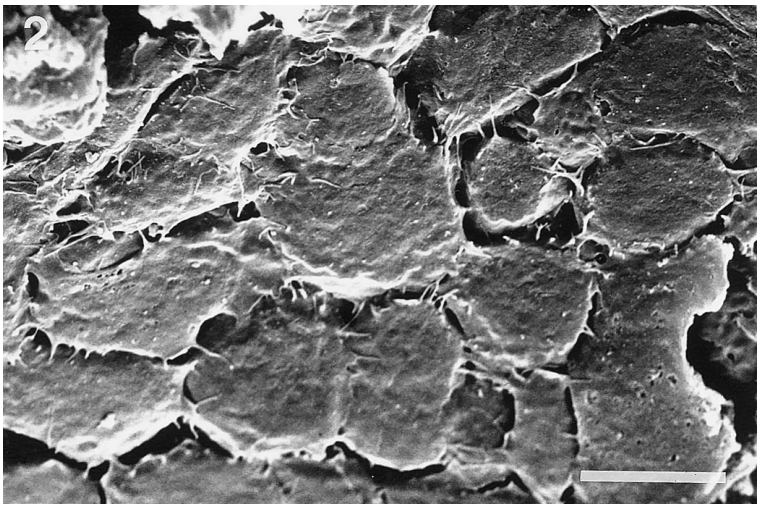

Fig. 2. EPC-cells producing a dense monolayer in multidish wells after 40 days incubation (11–12°C). SEM. Scale bar 10 μm.

point drying, the specimens were sputtered with gold and studied in a JEOL scanning electron microscope.

Results

Except for a few gyrodactylids containing mature embryos when collected and subsequently releasing their offspring in the wells, no reproduction of parasites was seen during the *in vitro* incubation. Parasites survived in water for up to 46 h, in culture medium for up to 118 h and in EPC-cell cultures for up to 139 h (table 1). *Gyrodactylus derjavini* attached to the monolayer by penetrating the EPC cells with the marginal hooklets in a way that could not readily be discerned from *in vivo* conditions. Scanning electron microscopy studies showed that EPC-cells 4 days post-inoculation showed numerous plasma membrane extensions during their colonization of the well (fig. 1). In contrast, monolayers 40 days old created dense pavement-like sheets (fig. 2). Due to the sterile conditions of the media, post mortem parasites retained their morphology as they were not subjected to bacterial degradation (fig. 3). However, after 40 days parasites were overgrown by EPC-cells (fig. 4) but it was still possible to discern the original parasite morphology in these parasites. After 55 days they gradually lost their integrity as the degradation of soft tissue was clearly advanced leaving only sclerotized parts such as marginal hooklets (fig. 5) and hamuli (fig. 6) with their original morphology. EPC-cells were tested for 19 enzyme reactions but only ten showed positive. No clear differences in

Fig. 3. *Gyrodactylus derjavini* after 40 days in sterile tissue culture medium. SEM. Scale bar 10 μm.

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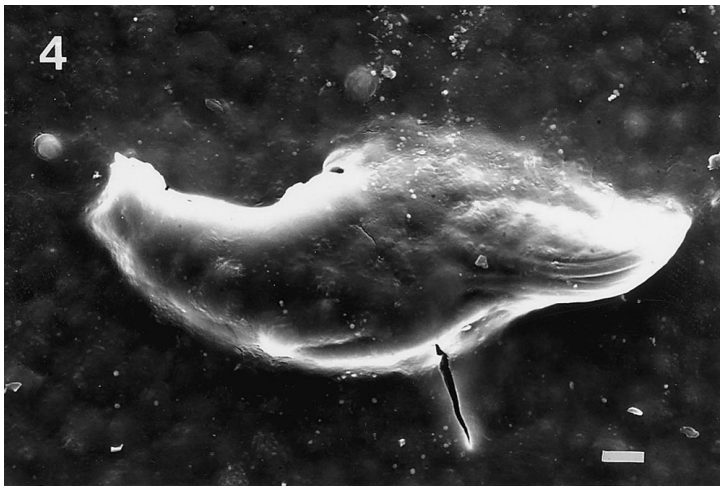

Fig. 4. *Gyrodactylus derjavini* encapsulated by EPC-cells after 40 days incubation. SEM. Scale bar 10 μm.

reactivity were recorded between the cell cultures with and without parasites (table 2).

Discussion

Although the microhabitat of ectoparasitic monogeneans is not only composed of epithelial cells but includes mucous cells and leukocytes as well, the present study showed that a monolayer of EPC epithelial cells will satisfactorily support attachment of *G. derjavini*. The stimuli released by these cells are evidently not sufficient to support reproduction of the parasites and *in vitro* propagation of this gyrodactylid clearly requires additional factors. It has previously been shown that monogeneans are able to survive detached from the host for a limited time period in water alone (Frankland, 1955; Buchmann & Bresciani, 1999) but no reproduction was seen under these conditions. Neither are cell culture media nor cell cultures with EPC-cells sufficient for a satisfactorily *in vitro* propagation although superior survival was observed in these media compared with water. However, the present work has shown that EPC cells are capable of encapsulating dead worms and subsequently to degrade them partially. This is noteworthy when evaluating the various host cell types and their interactions with pathogens. A number of investigations have indicated that host interactions with ectoparasites include a range of cell types but the relative

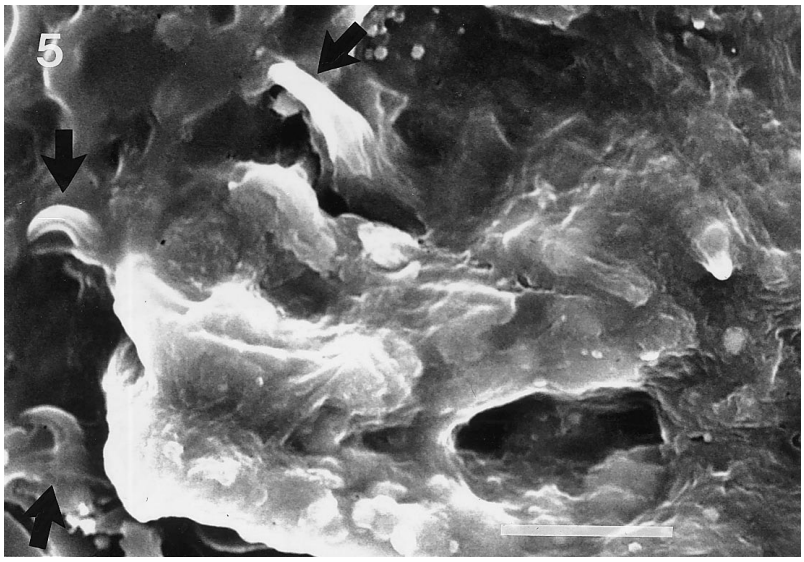

Fig. 5. Partially degraded opisthaptor of *Gyrodactylus derjavini* encapsulated in EPC-cells. Marginal hooklets can be discerned (arrows). SEM. Scale bar 10 μm.

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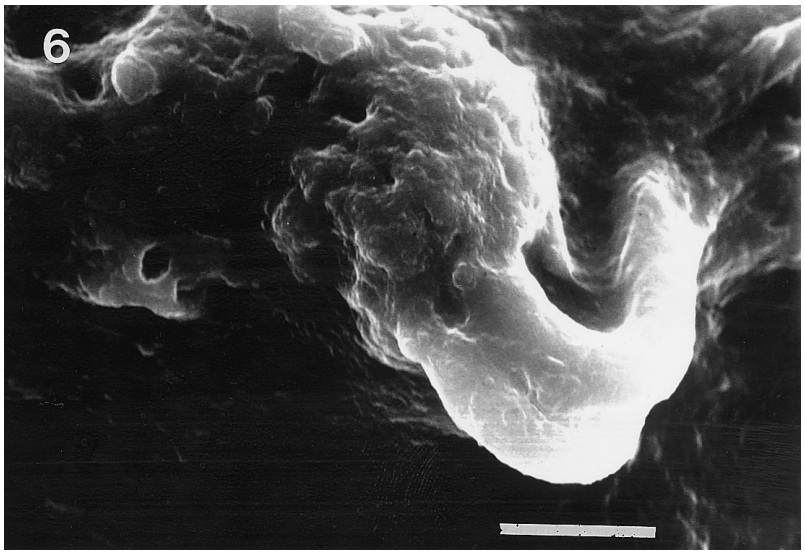

Fig. 6. Partially degraded opisthaptor of *Gyrodactylus derjavini* encapsulated in EPC-cells showing the two hamuli. SEM. Scale bar 10 μm.

importance of these factors have rarely been studied. Isolated macrophages were seen to elicit adverse effects on gyrodactylids (Buchmann & Bresciani, 1999) and it is suggested that intricate interactions between the various cell types in fish skin are activated upon contact with gyrodactylids (Buchmann, 1999). However, evidence has been presented which suggest that epithelial cells alone can interact with foreign material. Isolated Malpighian cells from salmon epithelia exhibited active migration and latex bead phagocytosis (Åsbakk & Dalmo, 1998) and the present study has shown interactions between a monogenean parasite and epithelial cells. Although the activity of EPC cells does not elicit any immediate adverse effects in *G. derjavini*, the independent activity of these cells is notable. However, this cell behaviour probably expresses a non-specific reaction which will occur following stimulation with any foreign material. In this light future investigations should further characterize combinations of various cell types and their interactions with monogeneans *in vitro*. Finally, it should be emphasized that EPC-cells represent a special carp epithelial cell line which probably differs from the epithelial cells found in the epidermis of trout, the natural host of *G. derjavini*. Therefore, future studies should also include the epithelial cells of trout.

Table 2. Enzyme reactions (ranked on a scale from 0: no reaction to 5: strong reaction) in EPC-cells with and without *Gyrodactylus derjavini*.

Enzyme	EPC-cells	EPC-cells with parasites
Alkaline phosphatase	2	1
Esterase	0.5	0.5
Esterase Lipase	0	0
Lipase	0	0
Leucin-arylamidase	4	4
Valin-arylamidase	2	3
Cystin-arylamidase	0	0
Trypsin	0	0
Chymotrypsin	0	0
Acid phosphatase	5	5
Phosphohydrolase	2	2
alpha-Galactosidase	0	0
beta-Galactosidase	1	1
beta-Glucuronidase	2	3
alpha-Glucosidase	0	0
beta-Glucosidase	0	0
Glucosaminidase	1	1
Mannosidase	0	0
Fucosidase	0.1	1

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