ELSEVIER

Fish & Shellfish Immunology 17 (2004) 95–103

Fish & Shellfish Immunology

www.elsevier.com/locate/fsi

Evidence of complement-mediated killing of *Discocotyle sagittata* (Platyhelminthes, Monogenea) oncomiracidia

Miguel Rubio-Godoy*, Robert Porter, Richard C. Tinsley

School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK

Received 15 August 2003; received in revised form 17 November 2003; accepted 17 November 2003

Abstract

Discocotyle sagittata oncomiracidia were rapidly killed when incubated in naïve plasma and immune sera from both rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), the killing proceeding at a faster rate with blood material from the latter fish species. The lethal activity of naïve plasma and immune sera was comparable. This was abolished after incubation at 45 °C for 30 min and by the addition of EDTA but not EGTA supplemented with Mg^{2+}, indicating that complement acting via the alternative pathway is responsible for the parasiticidal effect observed. Scanning electron micrographs showed varying degrees of surface disruption in larvae exposed to fish plasma, suggesting that complement acts by breaching the oncomiracidial tegument. Control (untreated) oncomiracidia showed no damage. Ultrastructural damage was more extensive in oncomiracidia exposed to brown trout plasma than to rainbow trout plasma for equal periods, suggesting that the complement cascade may be involved in mediating host susceptibility.

Keywords: Monogenea; *Discocotyle sagittata*; Trout; *Oncorhynchus mykiss*; *Salmo trutta*; Complement; Non-specific immunity; Scanning electron microscopy

1. Introduction

Monogenea are flatworm parasites of fishes. Teleost hosts have been shown to mount immune responses against monogeneans [1] and complement found in mucus has been proposed to mediate protective responses acting upon surface-dwelling parasites [1,2]. Indeed, complement acting via the alternative activation pathway was shown to kill *Gyrodactylus derjavini* [3,4] and *Gyrodactylus salaris* [5]. The lethal effect of complement has also been documented for the trematode *Schistosoma mansoni* [6,7], and through complement-fixing antibodies against the kinetoplastid *Cryptobia salmositica* in fish [8]. Additionally, complement has been proposed to mediate host specificity in several fish parasites, for example

* Corresponding author.
E-mail addresses: m.rubio-godoy@bristol.ac.uk, mrubiogodoy@yahoo.com (M. Rubio-Godoy).

1050-4648/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2003.11.005

C. salmositica [9,10] and *G. derjavini* [11]. Field studies of farmed fishes naturally infected with the monogenean *Discocotyle sagittata* have indicated that brown trout (*Salmo trutta* L.) usually present lower parasite intensities than rainbow trout (*Oncorhynchus mykiss* Walbaum) [12,13], suggesting that these fish species might differ in their susceptibility to the parasite. Experimental infection of both trout species has confirmed that *S. trutta* is innately less susceptible to *D. sagittata* than *O. mykiss* [14]. This investigation was undertaken to assess whether serum and complement act upon *D. sagittata* oncomiracidia and to determine if the differences between brown and rainbow trout in susceptibility to infection are associated, in part, with factors in host serum.

2. Materials and methods

2.1. Parasites

Freshly hatched (<1 h) *D. sagittata* oncomiracidia were obtained in the lab as described previously [15]. Briefly, parasite eggs were incubated at 13 °C for about four weeks and active infective larvae were recovered under a dissecting microscope after mass hatching induced by the onset of darkness [16].

2.2. Fish sera and plasma

Blood samples were obtained from naïve and parasitized rainbow trout (*O. mykiss* Walbaum) and brown trout (*S. trutta* L.). Immune sera were obtained in November 2001 during fieldwork in the Isle of Man, as described previously [13]. In short, class of the year, one- and two-year-old fish infected by *D. sagittata* were anaesthetised terminally with MS222 and bled. Blood was allowed to clot overnight at 4 °C and serum frozen (−70 °C) for transportation. Samples were thawed and aliquoted for antibody titre determinations; these aliquots were thawed and re-frozen several times—sera from the two salmonid species were subjected to the same procedures. For the incubation assays, a pool of five immune sera from one- and two-year-old fish known to have high anti-*D. sagittata* antibody titres [13] was used: separate incubations included sera which had been thawed only once and their thawed and re-frozen counterparts. To obtain naïve plasma of each trout species, three fish were anaesthetised and heparinized blood samples from the caudal vein were pooled and immediately centrifuged (14 000 × g, 3 min); naïve plasma was used in incubation assays within 1 h of extraction and discarded.

2.3. Incubation in fish sera and plasma

Active *D. sagittata* oncomiracidia were placed in PBS pH 7.0 at 4 °C in sterile 12-well culture plates (NUNC); for ease of monitoring, each well contained five or ten larvae in 1 ml solution. After controlling that no oncomiracidial mortality had occurred during manipulations, whole (immune and naïve) or heat-inactivated serum or plasma was mixed directly into each well to reach final dilutions of 1:20, 1:50, 1:100 and 1:200; this was considered time 0 for the incubation. Each dilution was tested on three replicates containing 25 oncomiracidia, and 30 control larvae were placed in PBS only. Plates were kept at 4 °C and mortality was quantified under a dissecting microscope at different time intervals. Living oncomiracidia swam vigorously, or moved or contracted spontaneously at regular intervals. Larvae were categorised as dead when immobile, opaque and showing signs of breakdown.

2.4. Inhibition of complement

Prior to use, serum and plasma samples were heat-inactivated by incubation at 45 °C for 30 min [4,5,17]. Additionally, fish plasma (1:20 dilution only) were inhibited with chelating agents: 5 mM ethylenediamine

tetraacetic acid (EDTA) or 5 mM ethyleneglycol tetraacetic acid (EGTA) + 1 mM $MgCl_2$ [5]; control incubations included EDTA or EGTA only.

2.5. Scanning electron microscopy

Oncomiracidia were incubated at 4 °C in 1:25 immune plasma and PBS only for controls, and reactions in individual wells were stopped at 15 min intervals by the addition of an excess of 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.2. Larvae were then post-fixed with buffered 1% osmium tetraoxide and washed in 0.1 M sodium cacodylate buffer prior to critical-point dehydration and gold sputtering for SEM analysis with a Philips 501B microscope.

2.6. Statistical analysis

Survival curves from different treatment groups were compared with Kaplan–Meier survival analysis (SPSS for Windows 10.0).

3. Results

D. sagittata oncomiracidia were killed rapidly when incubated with whole serum and plasma obtained from naïve or infected rainbow trout or brown trout. Infected fish have been shown to possess elevated anti-*D. sagittata* immunoglobulin titres; thus, sera from infected fish are immune. Fig. 1 illustrates the survival curves for oncomiracidia incubated in immune sera compared with untreated controls maintained in PBS at the same temperature. At the highest serum concentrations used, larvae started contorting violently and a halo-like coat of fuzzy material formed around them within minutes. Brown trout serum was more effective than rainbow trout serum in killing oncomiracidia at all concentrations tested, with no parasites surviving after 5 h at 1:200 dilution. A large proportion of larvae survived a 24-h incubation in 1:200 rainbow trout serum, and no mortality occurred in control wells. Killing was clearly serum concentration-dependent. Incubation in immune sera that had been thawed and re-frozen several times led to less steep mortality curves; however, after 2 h, 100% mortality was observed for 1:20 brown trout serum and 70% for 1:20 rainbow trout serum. Heat inactivation of immune sera prior to incubation markedly decreased their killing capacity, with 75–90% survival observed at all dilutions up to 24 h (data not shown; but similar to Fig. 2B). Although no significant mortality was observed in heat-inactivated immune sera incubations, oncomiracidia exhibited less motility than controls.

Incubation in freshly obtained naïve plasma also led to rapid mortality of oncomiracidia in a dose-dependent manner (Fig. 2A). Brown and rainbow trout plasma differed in their killing efficiency: for instance, 100% mortality was observed within 15 min in the two highest brown trout plasma concentrations tested, and within 30 min for 1:20 and 1.5 h for 1:50 rainbow trout plasma. After 8 h, 1:200 brown trout plasma had caused 96% mortality, and rainbow trout plasma 72%. A final data point at 24 h (not shown) recorded survival for only two groups: one oncomiracidium (4%) survived in 1:200 rainbow trout plasma, while 83% of controls were still active. Heat-inactivation abolished the killing ability of naïve plasma from both fish species, with 72–92% survival after 24 h (Fig. 2B); no significant difference between any of the survival curves was found. The addition of divalent cation chelators to incubation media adversely affected oncomiracidia, particularly EDTA, which killed most larvae within 2 h. However, their effect on plasma incubation could still be assessed based on two observations. First, the addition of 5 mM EDTA or EGTA temporally inhibited the killing ability of 1:20 naïve plasma from both fish species, extending maximal survival from 5–15 min in untreated plasma to up to 90 min in wells containing EDTA (Fig. 3). EDTA and EGTA incubations differed temporally, with a more rapid onset of mortality observed in the latter. Second,

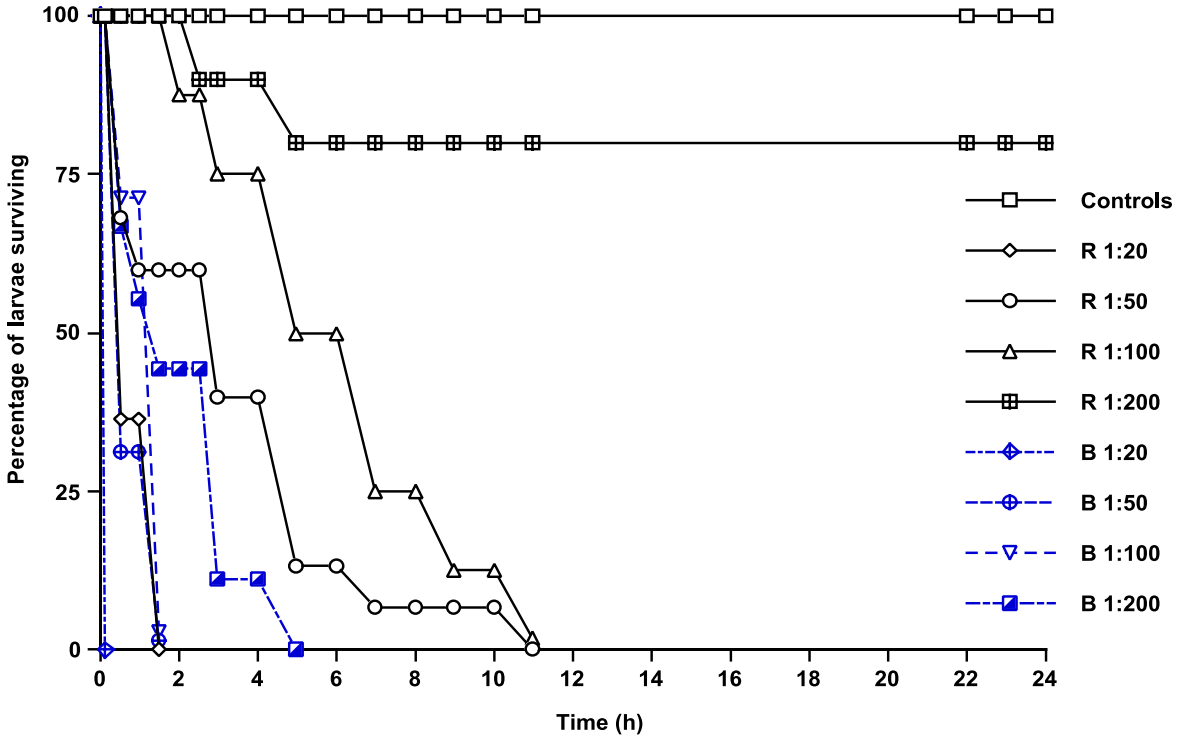

Fig. 1. Survival curves for *Discocotyle sagittata* oncomiracidia incubated in immune serum from farmed rainbow trout (*Oncorhynchus mykiss*) or brown trout (*Salmo trutta*) at 4 °C. R = rainbow trout serum; B = brown trout serum; ratios denote the serum dilution factor. Controls were incubated in PBS only at the same temperature.

the two chelating agents also differed qualitatively in terms of their effect: as was the case for untreated sera, a fuzzy coat formed around oncomiracidia incubated in the presence of EGTA, but not in the case of EDTA (Fig. 3). Incubation in EGTA only did not result in the formation of the fuzzy coat around dead oncomiracidia; and 40% survival was observed after 2 h, as opposed to 100% mortality within 30 min in the presence of sera + EGTA.

Representative samples of oncomiracidia incubated for 1 h in 1:25 fish plasma or PBS only are presented in Fig. 4. All control larvae incubated in PBS were swimming actively at the time of glutaraldehyde fixation and presented an intact appearance when viewed with SEM: cilia appeared undamaged (Fig. 4A) and the tegument had a regularly corrugated aspect (Fig. 4B). Oncomiracidia incubated in rainbow trout plasma for 1 h had lost their cilia (note the cells from which cilia have been shed in Fig. 4C) and their tegument presented a less-structured appearance (Fig. 4D). Incubation in brown trout plasma led to major damage to the oncomiracidia: the regular aspect of the tegument was lost (Fig. 4E) and this external layer was disorganized, blebbed and perforations presumably leading to the subtegumental layers were observed (Fig. 4F).

4. Discussion

This study demonstrates that oncomiracidia of the gill monogenean *D. sagittata* are sensitive to killing by whole sera or plasma from two salmonid host species in the UK. The experimental results point to

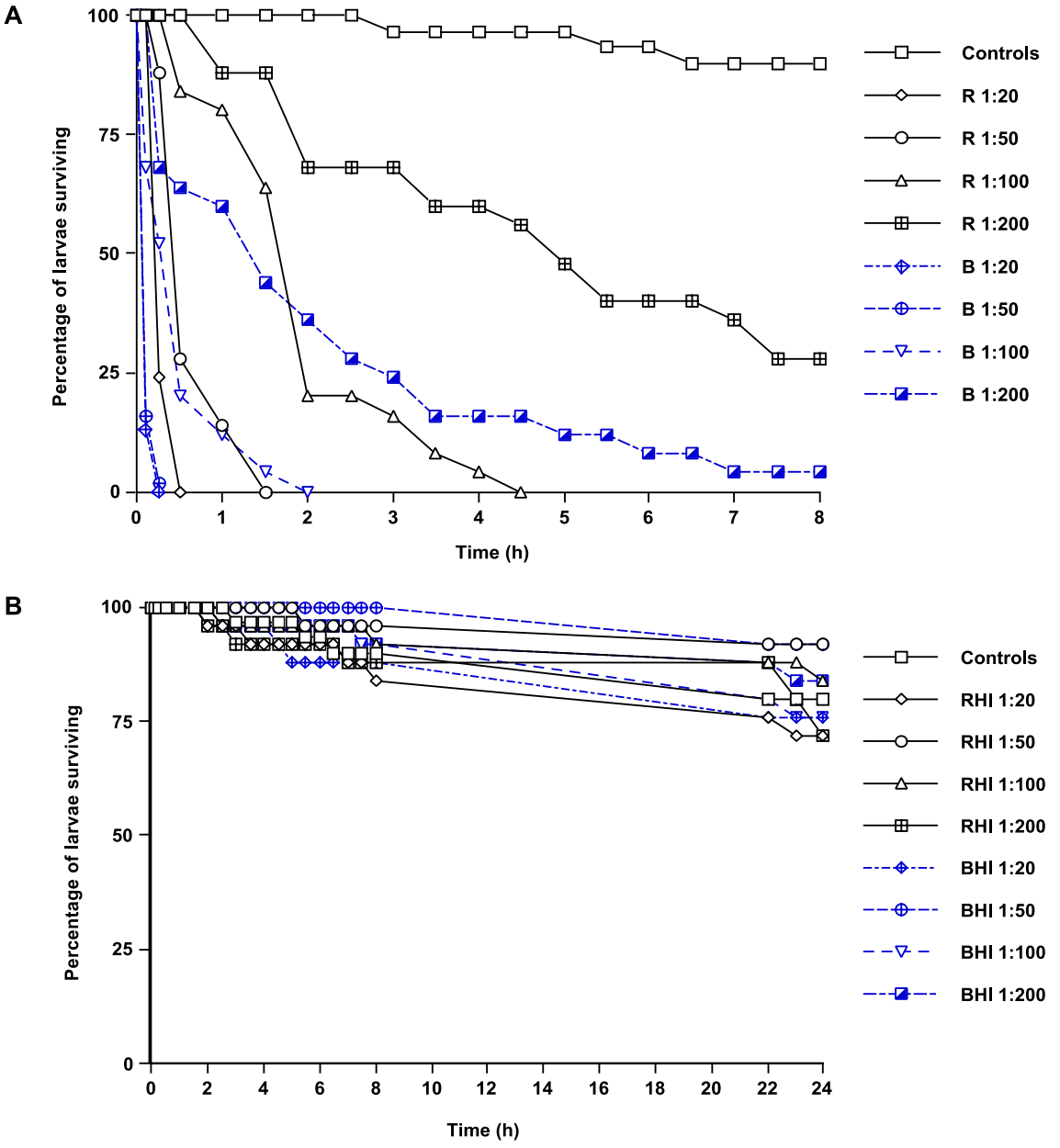

Fig. 2. Survival curves for *Discocotyle sagittata* oncomiracidia incubated in (A) whole or (B) heat-inactivated plasma from naïve rainbow trout (*Oncorhynchus mykiss*) or brown trout (*Salmo trutta*) at 4 °C. R = whole rainbow trout plasma; B = whole brown trout plasma; RHI = heat-inactivated rainbow trout plasma; BHI = heat-inactivated brown trout plasma; ratios denote the plasma dilution factor. Controls were incubated in PBS only at the same temperature.

complement being the main blood component responsible for the lethal effect observed. First, the parasiticidal activity in serum and plasma was abolished after incubation at 45 °C, a temperature that inactivates fish complement [17]. Additionally, immune sera gradually lost their killing ability when thawed and re-frozen several times, a procedure that would affect complement rather than immunoglobulin. Second,

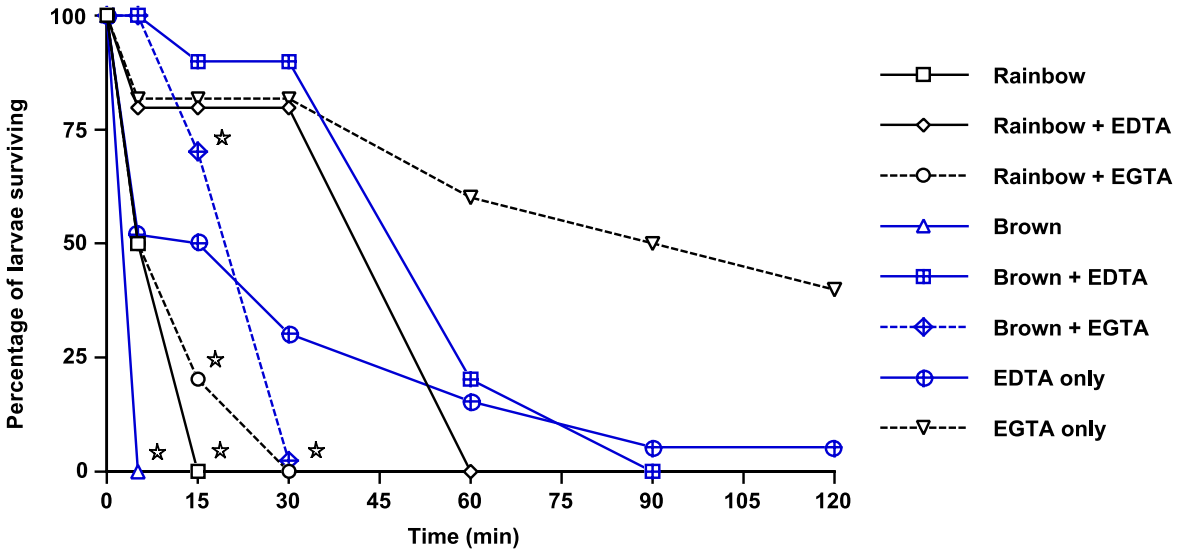

Fig. 3. Survival curves for *Discocotyle sagittata* oncomiracidia incubated in 1:20 dilution of plasma from naïve rainbow trout (*Oncorhynchus mykiss*) or brown trout (*Salmo trutta*) at 4 °C, in the presence or absence of 5 mM EDTA or 5 mM EGTA + 1 mM Mg^{2+}. Stars that denote fuzzy coats were detected around oncomiracidia.

divalent cation chelators modified the mortality dynamics of oncomiracidia exposed to plasma. Although in itself toxic to larvae, EDTA abolished the fast-acting killing properties of naïve plasma, extending survival from 5–15 min to up to 90 min. EGTA supplemented with magnesium ions proved less toxic to oncomiracidia but did not suppress the lethal effect of naïve plasma to the same degree as EDTA, with 100% mortality recorded after 30 min. Incubation in the presence of EGTA led to the formation of fuzzy coats around oncomiracidia, a phenomenon that was also detected in incubations with sera but not in the presence of EDTA + plasma nor of cation chelators alone. The accumulation of fuzzy material around dying parasites was likewise recorded during complement-mediated killing of *G. salaris* [5]. The different outcome of EDTA and EGTA + Mg^{2+} incubations indicates that the observed killing is mediated via the alternative pathway of complement [18]. The third evidence pointing to complement-mediated killing is that mortality dynamics did not differ markedly between incubations with naïve plasma and immune sera, suggesting the non-specific rather than specific nature of the lethal effect noticed. The rapid onset of mortality in naïve plasma at high concentrations suggests that pre-coating of parasites with antibodies would have no major effect in increasing killing efficiency. Taken together, these observations suggest that the alternative pathway of complement activation is responsible for the parasiticidal activity reported in this work. A lethal effect of complement has previously been shown for other parasites: for instance, the monogeneans *G. salaris* [5] and *G. derjavini* [3,4], and the miracidia of *S. mansoni* [6,7]. Killing of *Gyrodactylus* and *Schistosoma* was found to be mediated via the alternative complement pathway and was suspected to occur as a result of osmotic stress following damage to the tegument by immune attack complexes [3–7]. *Schistosoma* has recently been found to produce peptides that inhibit complement [19,20]. In the experiments we report, the involvement of the classical pathway cannot be completely excluded since naïve trout has detectable levels of anti-*D. sagittata* antibodies [13,21]. Thus, even in naïve fish plasma, oncomiracidia may be attacked by complement through both pathways: (1) larvae can be opsonized by naturally occurring immunoglobulin (provided they share determinants with adult worms from which the antigens for antibody titre determination were obtained [21]), which would activate the classical cascade; and (2) the alternative pathway could be initiated by C3 binding to the parasite, as has been shown for *G. derjavini* [4].

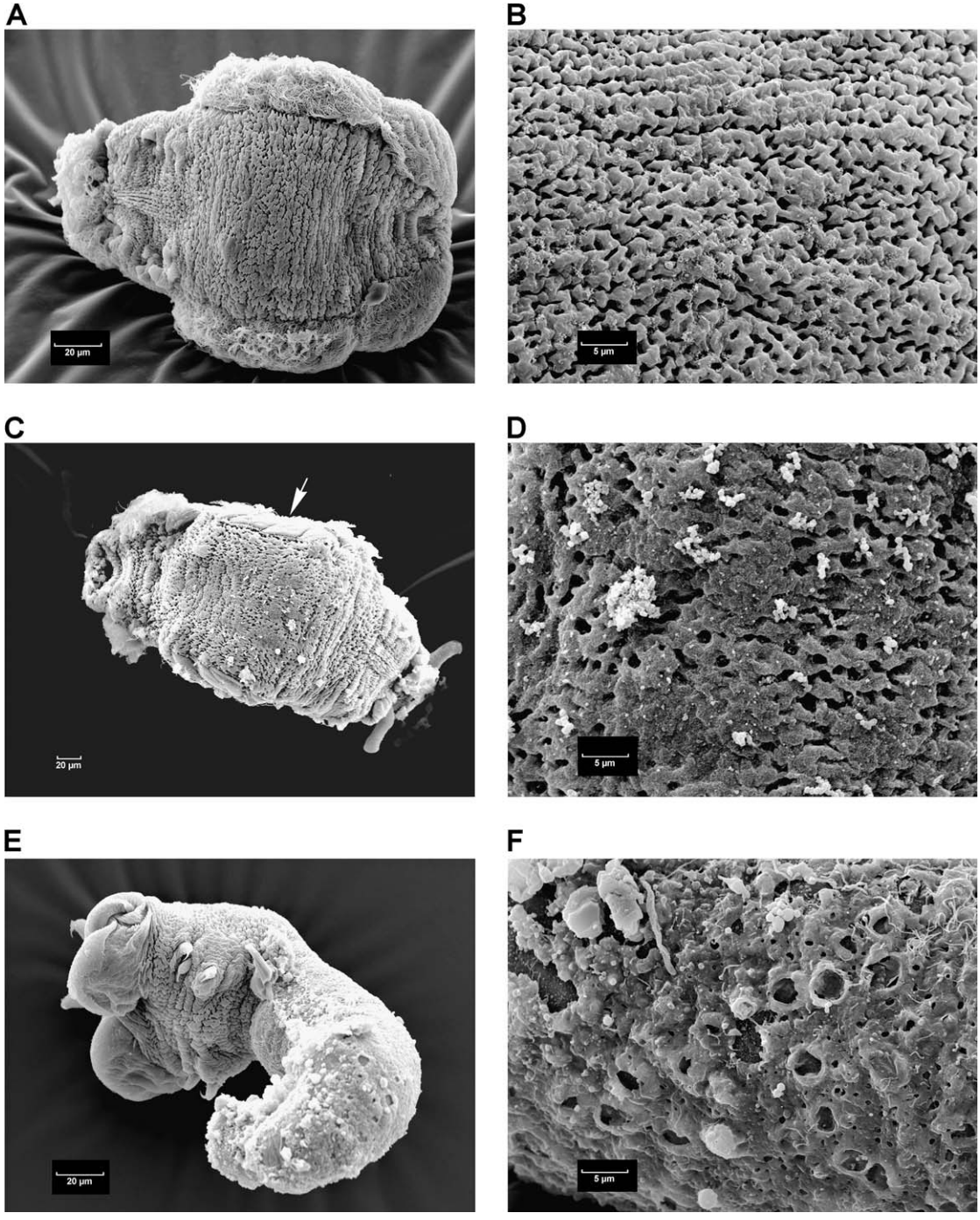

Fig. 4. Scanning electron micrographs of *Discocotyle sagittata* oncomiracidia incubated for 1 h at 4 °C in the presence or absence of fish plasma at 1:25 dilution. (A) Control oncomiracidium incubated in PBS only, and (B) regular aspect of its tegument at higher magnification; (C) oncomiracidium incubated in rainbow trout (*Oncorhynchus mykiss*) plasma (note arrow pointing to cells which have shed their cilia), and (D) higher magnification showing tegument irregularities; (E) oncomiracidium incubated in brown trout (*Salmo trutta*) plasma, and (F) close-up showing tegument disruption.

Oncomiracidia survived for up to 24 h when kept at 4 °C in PBS, and appeared structurally intact after 1 h as determined by SEM. Established *D. sagittata* withstand saltwater and adult worms have been recorded on sea trout [22]; the prolonged survival in PBS could imply that infective larvae are equally able to survive in brackish environments. In contrast, parasites exposed to plasma exhibited varying degrees of injury. In oncomiracidia exposed to brown trout plasma, large portions of the surface were disrupted, resulting in a disorganized and damaged tegument. Similar ultrastructural damage was observed after complement-mediated killing of *G. salaris* and *S. mansoni* with the loss of tegument and exposure of underlying muscle layer [3,7]. The authors suggested that tegumental disruption facilitated the action of lytic components of complement in the syncytium.

Brown trout and rainbow trout sera and plasma differed in their ability to kill *D. sagittata* oncomiracidia. This is interesting because *S. trutta* is more resistant to *D. sagittata* infection than *O. mykiss*, as suggested by a consistently lower prevalence and intensity of infection found in naturally infected farmed fish [12,13]; the different innate susceptibilities have been confirmed by controlled experimental infection of both species [14]. Complement-mediated destruction of parasites has been shown to correlate positively with innate susceptibility to several infections. For example, the innately resistant charr *Salvelinus fontinalis* (Mitchill) lyses the kinetoplastid *C. salmositica* via the alternative pathway of complement [23]. Susceptible rainbow trout (*O. mykiss*) can be effectively protected against this haemoflagellate through vaccination, protection being mediated by complement-fixing antibodies [8,23]. The biological relevance of the lethal activity of complement stems from the fact that this group of proteins is found in fish mucus ([2] and references therein). Indeed, it has been speculated that complement may, at least partially, mediate resistance to flagellates [9,10], gyrodactylids [5,11,24,25], and ciliates [25,26]. This study suggests that the difference in innate susceptibility to *D. sagittata* documented in brown trout and rainbow trout may be mediated in part by complement.

Acknowledgements

We thank John Ballard for access to fish and lab facilities in the Isle of Man, and Kurt Buchmann and Jens Sigh (Royal Veterinary and Agricultural University, Denmark) for their help and facilities to determine antibody titres. MRG is supported by postgraduate scholarships from the Mexican National Council for Science and Technology (CONACYT) and the Overseas Research Student Award Scheme (Universities UK).

References

[1] Buchmann K, Lindenstrøm T. Interactions between monogenean parasites and their fish hosts. Int J Parasitol 2002;32:309–19.
[2] Buchmann K. Immune mechanisms in fish skin against monogeneans—a model. Folia Parasitol 1999;46:1–9.
[3] Buchmann K, Bresciani J. Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*. Dis Aquat Org 1999;35:13–22.
[4] Buchmann K. Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). Dis Aquat Org 1998;32:195–200.
[5] Harris PD, Soleng A, Bakke TA. Killing of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) mediated by host complement. Parasitology 1998;117:137–43.
[6] Knopf PM, Petzke MM, McLaren DJ. Immobilization of *Schistosoma mansoni* miracidia by activation of the alternate pathway at unusually high serum dilution. Parasite Immunol 1993;15:325–37.
[7] Ouaissi MA, Santoro F, Capron A. *Schistosoma mansoni*: ultrastructural damages due to complement on schistosomula *in vitro*. Exp Parasitol 1980;50:74–82.
[8] Mehta M, Woo PTK. Acquired cell-mediated protection in rainbow trout, *Oncorhynchus mykiss*, against the haemoflagellate *Cryptobia salmositica*. Parasitol Res 2002;88:956–62.

[9] Forward GM, Woo PTK. An in vitro study on the mechanism of innate immunity in *Cryptobia*-resistant brook charr (*Salvelinus fontinalis*) against *Cryptobia salmositica*. Parasitol Res 1996;82:238–41.
[10] Woo PTK. Protective immune responses of fish to parasitic flagellates. Annu Rev Fish Dis 1996;6:121–31.
[11] Buchmann K, Uldal A. *Gyrodactylus derjavini* infections in four salmonids: comparative host susceptibility and site selection of parasites. Dis Aquat Org 1997;28:201–9.
[12] Gannicott AM. The biology of *Discocotyle sagittata* (Monogenea) infecting trout. PhD thesis. Bristol: University of Bristol; 1997. 312 pp.
[13] Rubio-Godoy M, Sigh J, Buchmann K, Tinsley RC. Antibodies against *Discocotyle sagittata* (Monogenea) in farmed trout. Dis Aquat Org 2003;56:181–4.
[14] Rubio-Godoy M, Tinsley RC. Comparative susceptibility of brown trout and rainbow trout to *Discocotyle sagittata* (Monogenea). J Parasitol, in press.
[15] Rubio-Godoy M, Tinsley RC. Trickle and single infection with *Discocotyle sagittata* (Monogenea: Polyopisthocotylea): effect of exposure mode on parasite abundance and development. Folia Parasitol 2002;49:269–78.
[16] Gannicott AM, Tinsley RC. Egg hatching in the monogenean gill parasite *Discocotyle sagittata* from the rainbow trout (*Oncorhynchus mykiss*). Parasitology 1997;114:569–79.
[17] Sakai DK. Repertoire of complement in immunological defense mechanisms of fish. Annu Rev Fish Dis 1992;2:223–47.
[18] Yano T. The nonspecific immune system: humoral defense. In: Iwama G, Nakanishi T, editors. The fish immune system: organism, pathogen and environment. London: Academic Press Ltd; 1996. p. 106–59.
[19] Inal JM, Schneider B, Armanini M, Schifferli JA. A peptide derived from the parasite receptor, complement C2 receptor inhibitor trispanning, suppresses immune complex-mediated inflammation in mice. J Immunol 2003;170:4310–7.
[20] Inal JM, Sim RB. A *Schistosoma* protein, Sh-TOR, is a novel inhibitor of complement which binds human C2. FEBS Lett 2000;470:131–4.
[21] Rubio-Godoy M, Sigh J, Buchmann K, Tinsley RC. Immunization of rainbow trout *Oncorhynchus mykiss* against *Discocotyle sagittata* (Monogenea). Dis Aquat Org 2003;55:23–30.
[22] Slinn DJ. Occurrence of *Discocotyle sagittata* on sea trout. Nature 1963;197:306.
[23] Woo PTK. Cryptobiosis and its control in North American fishes. Int J Parasitol 2001;31:566–74.
[24] Dalgaard MB, Nielsen CV, Buchmann K. Comparative susceptibility of two races of *Salmo salar* (Baltic Lule river and Atlantic Conon river strains) to infection with *Gyrodactylus salaris*. Dis Aquat Org 2003;53:173–6.
[25] Barker DE, Cone DK, Burt MDB. *Trichodina murmanica* (Ciliophora) and *Gyrodactylus pleuronecti* (Monogenea) parasitizing hatchery-reared winter flounder, *Pseudopleuronectes americanus* (Walbaum): effects on host growth and assessment of parasite interaction. J Fish Dis 2002;25:81–9.
[26] Buchmann K, Sigh J, Nielsen CV, Dalgaard M. Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*. Vet Parasitol 2001;100:105–16.