In vitro interactions between Neoparamoeba sp. and Atlantic salmon epithelial cells

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Abstract

Neoparamoeba sp., including the putative aetiologic agent of amoebic gill disease in cultured fish (N. pemaquidensis), were incubated in vitro with an Atlantic salmon gill epithelium (RGE-2) cell line. Proliferation by the amoeba population was dependent upon culture osmolarity; no growth occurred at 330 mM kg\(^{-1}\) but a sixfold increase was observed at 1000 mM kg\(^{-1}\). At 780 mM kg\(^{-1}\) there was a fourfold increase in the amoeba population but a concurrent decrease in RGE-2 cell density that was significantly greater than that caused by the high culture osmolarity alone. This apparent cytopathic effect (CPE) developed rapidly and resulted in complete cytolysis of the monolayer in 5 days. CPE occurred in multiple foci and presented as cell vacuolation, rounding and clumping, and the rapid clearance of large areas of the cell monolayer. The possibility that CPE is because of the presence of Neoparamoeba sp. derived cytolytic products is discussed in the context of the pathology of the disease in vivo and the occurrence of secreted cytopathogenic compounds in other amoeba species.

Keywords: cytolysis, cytopathic effect, fish cells, in vitro, Neoparamoeba sp., proliferation.

Introduction

Neoparamoeba pemaquidensis is the putative aetiological agent of amoebic gill disease (AGD) in farmed fish (Dykova, Figueras & Peric 2000). It is a free-living amoeba that for undetermined reasons parasitises Atlantic salmon, Salmo salar L., coho salmon, Oncorhynchus kisutch (Walbaum), Chinook salmon, O. tshawytscha (Walbaum), rainbow trout, O. mykiss (Walbaum), turbot, Scophthalmus maximus (L.), seabass, Dicentrarchus labrax (L.), and sharpsnout bream, Diplodus puntazzo L., in culture (Munday, Zilberg & Findlay 2001). The pathology caused is typical of a host inflammatory response with leucocyte infiltration, lamellar fusion, hyperplasia and hypertrophy of the gill epithelium (Munday, Foster, Roubal & Lester 1990; Adams & Nowak 2001). Tissue damage is not consistent with cytolytic events that are the main mechanism of pathogenicity seen in amoebic conditions in mammals (Visvesvara & Stehr-Green 1990; Alizadeh, Pidherney, McCulley & Niederkorn 1994; Taylor, Pidherney, Alizadeh & Niederkorn 1995). These cytopathic effects (CPE) are for the most part the result of proteolytic enzymes causing direct cytolysis or stimulating apoptosis of cells in the infected tissue. The nature of the CPE of these organisms can be modelled in vitro using cell cultures as the target tissue (John & John 1989). The cytopathogenicity of gill derived Neoparamoeba sp. for cultured cells was tested in this way with the aim of determining if these amoebae produce cytolytic products, the effects of which may be masked in vivo by the host tissue response.

Materials and methods

Amoebae

Amoebae were harvested from gills of Atlantic salmon affected by AGD maintained within the National Key Centre for Aquaculture aquarium, University of Tasmania, Launceston. Fish showing
AGD pathology (white gross gill lesions) were killed by anaesthetic overdose (Aquasafe®), the entire gill basket removed and transported to the laboratory in sterile sea water containing antibiotic and antifungal solution [5% v/v 5000 IU mL⁻¹ penicillin and 5 mg mL⁻¹ streptomycin solution (Sigma, Sydney, Australia), 1% v/v 10 mg mL⁻¹ gentamycin (Sigma) and 0.25 mg mL⁻¹ amphotericin B (Invitrogen, Mount Waverley, Victoria, Australia)]. The medium was exchanged for sterile distilled water and the gills mixed for 5 min to loosen the attachment of amoebae to tissue. Gill filaments were scraped into a Petri dish using cell scrapers to remove mucus and surface epithelial cells. The suspension was centrifuged at 400 g for 10 min and the pellet resuspended in sea water. The suspension was further diluted with sterile sea water to approximately 50 times and 30 mL decanted into multiple Petri dishes. Amoebae were allowed to adhere to the surface of the dishes for 1 h, following which the medium was decanted into fresh Petri dishes to undergo a second round of adherence. Adhered amoebae were resuspended by the addition of 3 mL trypsin-ethylene-diaminetetraacetic acid (EDTA) solution (0.25%) (Invitrogen) for two minutes, then pooled and diluted with sterile sea water prior to centrifugation at 400 g for 10 min. Pelleted amoebae were resuspended in sea water and the viable population estimated using a haemocytometer and trypan blue (Phillips 1973). The proportion of Neoparamoeba sp., including N. pemaquidensis, in the population was measured by an indirect immunoperoxidase method using a primary rabbit anti-N. pemaquidensis (PA 027) antibody using the method of Bridle, Butler & Nowak (2003).

RGE-2 cell line

The RGE-2 cell line was established from primary cultures of gill tissue from Atlantic salmon at the School of Aquaculture, University of Tasmania. It has an epithelial morphology that forms confluent monolayers approximately 15 days post-passage. Cultures were maintained at an osmolarity of 330 mM kg⁻¹ in a standard culture medium [Leibovitz (L-15) medium supplemented with 10% foetal calf serum (FCS), 2 mM l-glutamine (Invitrogen), 1% v/v penicillin streptomycin solution (Sigma) and 1% v/v 30 mM NaCl]. Cells were passaged at a split ratio of 1:3 by the addition of trypsin-EDTA (Invitrogen). All cultures used in these experiments were around 95% confluent with a cell density of approximately 2.8 × 10⁶ cells mL⁻¹.

Incubation of amoebae with epithelial cells

RGE-2 were passaged into 12 well tissue culture plates (Nunc) and allowed to reach confluence with standard culture medium. Media were removed and the monolayers washed three times with 0.01 M phosphate buffered saline (PBS) pH 7.4 to remove traces of FCS. Isolated amoebae were resuspended to a final density of 1 × 10⁶ cells mL⁻¹ in either serum free standard culture medium (330 mM kg⁻¹), hyperosmotic culture medium (780 mM kg⁻¹), or seawater culture medium (1000 mM kg⁻¹). Hyperosmotic medium was prepared by the addition of 5% v/v salt solution (50% v/v 5 M MgCl₂, 50% v/v 2.5 M Na₂SO₄, pH 7.4) and 5% v/v sugar solution (33.3% v/v 1 M sorbitol, 33.3% v/v 1 M mannitol and 33.3% v/v 1 M xylose, pH 7.4) to serum free standard culture medium. Seawater culture medium was prepared by the addition of 7.5% v/v salt solution and 7.5% v/v sugar solution to serum free standard culture medium. Two hundred microlitres of amoeba suspension was added to appropriate wells of culture plates and incubated at 18 °C. Control wells consisted of cells maintained without amoebae with either standard, hyperosmotic or seawater culture media. Amoeba growth estimates were measured daily by counting amoebae per field of view in five random fields in duplicate culture wells (at ×200 magnification). RGE-2 cell survival was measured using a modified MTT assay. Briefly, media were removed from four replicate wells, washed with PBS to remove amoebae and 100 μL of 5 mg mL⁻¹ MTT in L-15 added and incubated at 18 °C for 4 h. Media were removed and 100 μL dimethyl sulphoxide was then added, the contents mixed by pipetting for 10 min and the supernatants read at an optical density of 550 nm on a Spectra Rainbow thermo-microplate reader (Tecan, Cleveland, Queensland, Australia). A standard curve was produced by plotting haemocytometer cell counts against the corresponding OD.

Statistical analyses

Treatments were compared through time in a series of paired contrast analyses of covariance. Pairs were defined a priori. Results were considered statistically significant if P ≤ 0.05.
Results

Amoeba growth

At 330 mm kg$^{-1}$ there were no significant changes in the amoeba population when incubated with or without RGE-2 cells ($F = 0.712$, d.f. 1144, $P = 0.40$) (Fig. 1). In this situation viable amoebae were observed, as determined by a trypan blue exclusion assay, but these cells were rounded and displayed no pseudopodia. When incubated in hyperosmotic medium at 780 mm kg$^{-1}$ there was an approximate threefold increase in the amoeba population. Amoebae incubated with epithelial cells showed significantly greater population growth over time than without cells ($F = 17.461$, d.f. 1144, $P < 0.001$). Amoebae incubated with seawater culture medium at 1000 mm kg$^{-1}$ showed a sixfold increase in population size with RGE-2 cells and a fourfold increase without cells; a rate of growth that was significantly different ($F = 16.84$, d.f. 1144, $P < 0.01$). Growth over time of these amoebae was significantly greater than amoebae maintained at 780 mm kg$^{-1}$ both with RGE-2 cells ($F = 16.665$, d.f. 1144, $P < 0.001$) and without cells ($F = 12.34$, d.f. 1144, $P < 0.05$).

Effects of amoeba incubation on epithelial cells

When amoebae were incubated with RGE-2 cells with standard culture medium there was no significant effect on cell survival or cell morphology and the monolayer maintained its integrity and confluency (Fig. 2). Incubation of amoebae with RGE-2 cells at 1000 mm kg$^{-1}$ resulted in a rapid degradation of the cell monolayer that was complete by 5 days post-incubation. Similarly, control media at this osmolality caused rapid cell death that masked any effects of amoeba incubation and subsequent experiments were not performed at this osmolality. Control media at 780 mm kg$^{-1}$ did not significantly affect RGE-2 survival until day 9, following which there was a rapid degradation of the monolayer. The addition of amoebae to RGE-2 cells at this osmolality resulted in rapid breakdown of the cell monolayer. Cell survival was 50% by day 3 and total monolayer degradation occurred by day six. This rate of cell death was significantly greater than that caused by control media at 780 mm kg$^{-1}$ alone ($F = 11.52$, d.f. 192, $P < 0.001$). These apparent CPE developed in multiple foci causing vacuolation and rounding and clumping of cells that became highly light refractive and detached from the culture surface leading to large areas of clearing within the monolayer (Fig. 3). Cell lysis in control cultures at 780 mm kg$^{-1}$ was uniform and did not occur in the same focal pattern; similarly the degree of vacuolation was not as evident in these cultures, although cell rounding and clumping did occur. At 330 and 780 mm kg$^{-1}$ amoebae were predominantly seen associated with, but not attached to, the surface of the cell cultures. At 780 mm kg$^{-1}$ some pseudopodial formation was observed but for the most part amoebae appeared flattened and polygonal and were distinct from the rounded amoebae seen at the lower osmolality. At 1000 mm kg$^{-1}$ amoebae attached to the culture surface were similarly flattened and

![Figure 1](#) Changes in Neoparamoeba sp. population density following incubation at 330, 780 and 1000 mm kg$^{-1}$ with and without RGE-2 cells. Data are mean number of amoebae per field of view in 10 random fields ± SD.
study causative cytopathogenic products have been collected and characterized for the main disease causing species (Visvesvara & Stehr-Green 1990; Alizadeh et al. 1994; Taylor et al. 1995). AGD is the most significant amoebic condition affecting farmed fish, but the pathology of these infections is not typical of mammalian amoebiasis in that there is no evidence of cytolytic activity against host tissue when viewed in light, TEM or SEM preparations. Typically, histological sections of AGD lesions show intact proliferating cells in close association with amoebae and extensive host inflammatory responses in areas of amoeba infected gill. However, we have demonstrated that at least in vitro, Neoparamoeba sp. is able to cause CPE in cultured gill epithelial cells that culminates in cytolysis. Neoparamoeba sp. cytopathogenicity against RGE-2 cells was measured at an osmolarity of 780 mM kg\(^{-1}\) which is osmotically sub-optimal for this organism, and hyperosmotic for cultured cells. This environment represents a compromise position that appears to be metabolically suitable for the amoebae, which are able to proliferate, and for RGE-2 cells, which remain viable for more than 7 days, after which their morphological characters and survival begin to change. However, the potential for artefactual cytolytic activity because of the compromised osmoregulation of both amoebae and cells, and the lack of cytolytic pathology in vivo, necessitates that the measured CPE of cultured cells in vitro be further investigated to determine whether this is biologically relevant to the pathogenicity of Neoparamoeba sp. A further complicating factor is the presence of other amoebae within the in vitro inoculum. Immunohistochemistry consistently identified positively stained cells as Neoparamoeba sp. within the inoculum at >98% of the total amoebae. The nature and the possible effects of the remaining 2% is much studied but remains largely unknown. What is understood is that Neoparamoeba sp. and more specifically N. pemaquidensis are consistently identified as the largest group of amoebae present on the gills of AGD affected fish and within the inocula used in this study. The assumption we make therefore is that the observed cytolysis in vitro is attributable to the largest amoeba population, i.e. Neoparamoeba sp.

In vitro CPE caused by amoebae is well documented, especially for Acanthamoeba spp. and Naegleria spp. (De Jonckheere 1980; John & John 1989). The pattern of CPE is different between amoeba species, but typically cytolysis is the end
point and can occur in as little as 2 days following incubation (De Jonckheere 1980). Such methods have been used to discriminate between pathogenic and non-pathogenic amoeba species and strains, but often the reproducibility of CPE assays is low (De Jonckheere 1980). This author measured the CPE

Figure 3 Cytopathic effects observed in RGE-2 epithelial cells following the incubation with *Neoparamoeba* sp. at 780 mg kg⁻¹. (a) Cell monolayer prior to addition of amoebae; (b) 2 h post-incubation with amoebae; (c) 8 h post-incubation; (d) 12 h post-incubation; (e) 24 h post-incubation; (f) 48 h post-incubation (bar = 50 μm).
of 36 species and strains of Acanthamoeba spp. and found that temperature had a significant influence on the rate of cytolytic activity. However, whilst an increase in temperature from 30 to 37 °C increased cytolytic activity of most species, in some species and strains of the same species, the opposite was the case, highlighting the variability when measuring these responses in this type of assay. The incubation of Neoparamoeba sp. with cultured epithelial cells was replicated four times and produced consistent cytolytic outcomes at the constant temperature of 18 °C; complete CPE occurred by day 5 post-incubation. The effect of incubation at different temperatures was not measured and further investigation should monitor the influence of temperature on these effects, particularly since laboratory infections with Neoparamoeba sp. in fish are more effective at causing AGD within a narrow temperature window of 14–17 °C (B. F. Nowak, unpublished data).

In addition to limited reproducibility and the influence of temperature, the in vitro cytopathogenicity of amoebae is also dependent upon the target cell used. For example, Naegleria fowleri is cytopathogenic for mammalian lung, kidney, foreskin, ovary, connective tissue, neuroblasta and carcinoma cell lines, but the African green-monkey kidney (Vero) cell line has proven to be the most pathogenic for mammalian lung, kidney, foreskin, cell used. For example, the apparent absence of cytolysis is the causative agent of AGD and data that refute this have not been produced. It is more likely then that N. pemaquidensis, like the majority of other disease causing amoebae currently under study do produce compounds of a cytolytic nature. In vivo evidence for this is difficult to collect because of the host tissue disturbances that occur at the site of amoeba attachment, but in the absence of host immune responses in vitro, the cytolytic effects of these compounds are more easily seen.

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