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In vitro culture of the avian echinostome *Himasthla elongata*: from redia to marita

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Abstract

Axenic primary cultures of *Himasthla elongata* rediae harvested from hepatopancreas of naturally infected marine prosobranch snail *Littorina littorea* were maintained in Leibovitz's L-15 medium (osmolarity of approximately 780 mOsm, pH 7.8, temperature 14 °C under normal atmospheric conditions). Cultured rediae were active, motile and demonstrated high synthetic activity in metabolic labelling experiment. Long-term cultivation experiment showed 50% survival level of the rediae for up to 70 days and significant differences between mortality in redia groups derived from different host individuals. Half of the rediae in the most robust group survived for up to 163 days, when the experiment was terminated. Development and emergence of in vivo preformed cercariae and daughter rediae was observed. Cercariae in the culture also encysted, transformed into metacercariae and some of them in one to two weeks after the transformation spontaneously excysted into juvenile maritae. The employed culture system is characterized by a very low level of proteolytic activity. This system is suggested as a method permitting to obtain rediae secretory–excretory products free of host-derived contaminants.

Index Descriptors and Abbreviations: Himasthla elongata, Trematoda, redia, cercaria, metacercaria, marita, *L. littorea*, Gastropoda, in vitro, axenic primary cultures; SEP, secretory–excretory products; SSW, sterile seawater; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PADK, protease activity detection kit; BSA, bovine serum albumin.

1. Introduction

In vitro cultivation of parasitic helminths, including the digenetic trematodes, has long been a valuable tool in providing insights into naturally complex host–parasite interactions (Coustau and Yoshino, 2000). Success in culturing some trematode species in synxenic systems made possible the investigation of mechanisms of interactions between a larval parasite and host humoral factors, effector cells, and tissues (Coustau et al., 1997; Laursen and Yoshino, 1999; Loker et al., 1999; Sapp and Loker, 2000a; Sapp and Loker, 2000b; Yoshino and Laursen, 1995). However, in order to study precise mechanisms allowing larval trematodes to interact with snail defense system, it is essential to establish axenic in vitro culture system, ideally one that is chemically defined and capable of providing parasite secretory–excretory products (SEP) that are free of host-derived contaminants (Bixler et al., 2001; Coustau and Yoshino, 2000). This approach gives excellent possibility to identify active components of SEP, determine their targets and, in general, to control experimental conditions more accurately (Connors and Yoshino, 1990; Connors et al., 1991; Crews-Oyen and Yoshino, 1995; Lodes and Yoshino, 1990; Lodes et al., 1991; Loker et al., 1992). These two methods compliment each other. However, up to date only a very small array of experimental models has been used. Only several members from three digenean families of medical and veterinary significance—Schistosomatidae, Echinostomatidae, and Fasciolidae, infecting only basommatophoran pulmonates (*Biomphalaria*, *Bulinus*, *Lymnaea*)—have been under in vitro investigations.

To gain a better insight into mechanisms that govern snail/trematode compatibility and to trace its evolu-

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tionary aspects, it would be helpful to use comparative approach and to include in the analysis additional models, in which the host is phylogenetically distant from pulmonates. Taking into account that specificity level seems to have been changing in the course of mollusc–digenean co-evolution (Gibson and Bray, 1994), investigation of systems more archaic than pulmonate-based ones is very reasonable. In this context, the marine prosobranch snails are of special interest because, firstly, basic properties of their internal defense systems differ from that of pulmonates (Yakovleva et al., 2001) and, secondly, in some cases their trematode parasites have well-studied relatives infecting pulmonate hosts. The last point is especially important for adequate comparative analysis.

Intramolluscan development of the marine echinostome avian fluke, *Himasthla elongata*, is similar to that of other members of family Echinostomatidae. It involves the sequential production of several distinct generations including *Littorina littorea*—infective miracidia, primary or mother sporocysts, mother and daughter rediae and cercariae, the infective larvae for the intermediate host—blue mussel, *Mytilus edulis*. Definitive host, seagulls, become infected by eating mussels with encysted metacercariae (Werding, 1969). Here we report the primary in vitro culture of *H. elongata* rediae maintained in axenic conditions. This is the first reported cultivation of trematodes infecting marine prosobranch mollusc.

2. Materials and methods

2.1. Parasite isolation and in vitro cultivation

Littorina littorea snails were collected on the seashore near the Biological Station "Kartesh" (Chupa inlet, Kandalaksha bay of the White Sea). Infected periwinkles (shedding *H. elongata* cercariae) 5–20 years old were used in experiments as a source of rediae. Infected snails were kept in cages until used and supplied with food (fucoid seagrass) ad libitum.

Prior to dissection snails were washed and placed for two days into aquariums filled with filtered seawater (salinity 24‰). After digestive tract of snails has emptied, periwinkles were carefully crushed, their bodies placed in petri dishes filled with sterile sea water (SSW) and rediae were harvested from host hepatopancreas under dissection microscope. Pool of rediae sampled from one host individual was termed "clone." Isolated rediae were transferred to fresh SSW and carefully rinsed to remove snail debris. Then, after 10 abundant washes in SSW containing 100 μg/ml gentamycin, the parasites were placed into a well of 24-well tissue culture plates for suspensions (Sarstedt) containing 1 ml of Leibovitz (L-15) culture medium (800 mg L-15 powder, 20 ml distilled water, 80 ml SSW, 1 ml penicillin–streptomycin–neomycin solution; ~780 mOsm; pH 7,8), sterilized using 0.1 μm filter units (Millipore). All reagents were from Sigma. Cultures were incubated at 14 °C under normal atmospheric conditions and the medium was changed once in three days. Monitoring of cultivation events was performed daily using inverted microscope. Altogether, about 50 redial "clones" were monitored in the course of two- and three-week cultivations.

2.2. Long-term survival experiment

Himasthla elongata redial microhemipopulation (up to about 1000 individuals) from one host individual was large, which allowed to perform the comparison of their long-term survival between the "clones." From 30 to 160 rediae of one "clone" in 1 ml of the medium were placed into each of five wells in 24-wells flat-bottom plate and cultured as described above. Three redial "clones" (954 individuals) were used in the experiment. Once in 2–5 days rediae condition was examined and surviving was assessed for each redia in the course of 163-days experiment.

2.3. Identification of rediae metabolic activity

In order to verify in vitro synthetic activity of *H. elongata* rediae, their newly produced proteins were labeled metabolically by incorporation of [^{14}C] amino acids mixture (specific activity >500 Ci/mM). The rediae were maintained in 96-well culture plates (Sarstedt) (250 in each well) and cultured as above. Cultures were pulsed for 24 h at the first and the sixth day of cultivation with ^{14}C-amino acids mixture (100 μCi/ml), rediae were sampled and revealed with SDS–polyacrylamide gel electrophoresis (12% gels) (Laemmli, 1970). The slab gel was stained with ammoniacal silver (Wray et al., 1981), dried and exposed to X-ray film for 60 days at −20 °C.

2.4. Identification of the medium proteolytic activity

To estimate possible toxicity of the medium that was in contact with rediae (rediae-conditioned medium), one of the potentially toxic characteristics, total protease activity accumulated for 48 h, was evaluated using Protease Activity Detection Kit (PADK: PanVera Corporation, USA). Four hundred rediae were placed into each of eight wells of 24-well plate filled with 800 μl medium and cultured as above. Four replicates of the media were collected at the fourth and the sixth day following the culture initiation after 48 h exposure and after centrifugation at 10,000*g* for 10 min supernatants were used as samples of redia-conditioned medium. Pure medium was used as a blank. The samples and blanks

were processed according to the PADK protocol and read on a Microwell strip reader EL 301 at A_{490}. Also four replicates of positive control, which was supplied in the kit, were included in the assay.

2.5. Data analysis

The arcsin-square root transformed data from survival experiment were analyzed with help of the statistical analysis software Statistica for Windows v5.5. Means of survival in the course of the cultivation were compared using one-way ANOVA. Confidence intervals (95%) of means were retransformed after computation.

3. Results

Once transferred in the medium, *H. elongata* rediae showed vigorous bending movements as well as longitudinal and transverse contractions. Rediae motility was maintained throughout the entire period of cultivation (Fig. 1A). Dead or dying rediae were easily identified by their loss of motility and tegument integrity. Long-term cultivation experiment showed 50% survival level for up to 70 days (Fig. 2) and highly significant between-"clone" differences in survival rate (ANOVA: $F_{(2)} = 212.5$, $P \ll 0.001$). To note, 50% of the rediae in the most robust "clone" survived for up to 163 days, when the experiment was terminated. During cultivation, moribund or dead cercariae, their lost tails and dead rediae bodies (Fig. 1B) were the only source of food for the rediae. In no case were rediae observed to attack one another. No somatic growth was noted.

In the course of cultivations, a great quantity of motile cercariae were released during the first week after the initiation of cultures. Once the main pool of predeveloped larvae has been released, cercariae production gradually decreased, but continued until end of all cultivations. Newly born larvae seemed normally developed and showed normal swimming and crawling behavior. Despite the fact that a large part of *H. elongata* motile larvae underwent elimination with changed medium, some of the residuary cercariae after about 24 h of activity rounded off, encysted and transformed into metacercariae. In very rare cases cercariae were observed to encyst inside moribund rediae (Fig. 3A). As a rule, encysted larvae showed sluggish and rare muscle contractions and were like that until the cultures were terminated. However, some of them in one to two weeks after the transformation became spontaneously

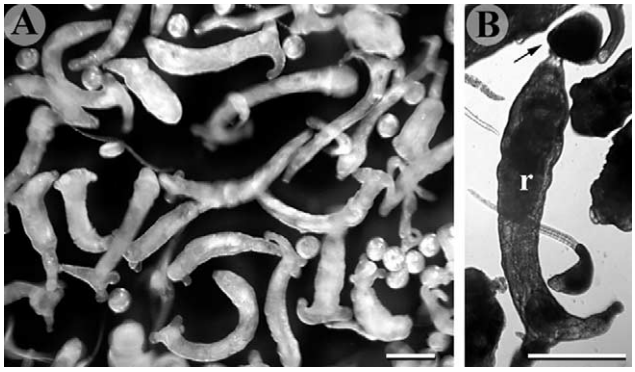

Fig. 1. In vitro cultivation of *H. elongata* rediae. (A) General view of culture after 40 days of cultivation; (B) ingestion of dead neighbor (arrow) by redia (r). Scale bar = 500 μm.

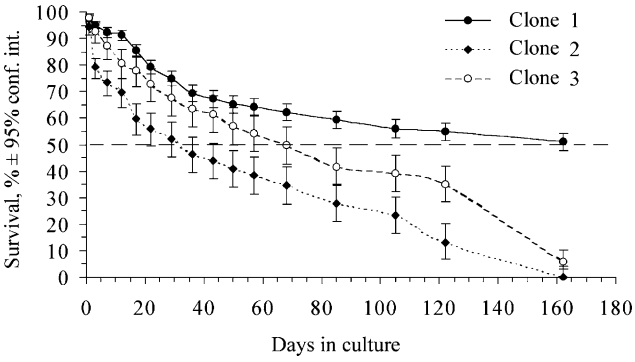

Fig. 2. Survival dynamics in three "clones" of rediae in the course of long-term survival experiment; dotted line—level of 50% survival.

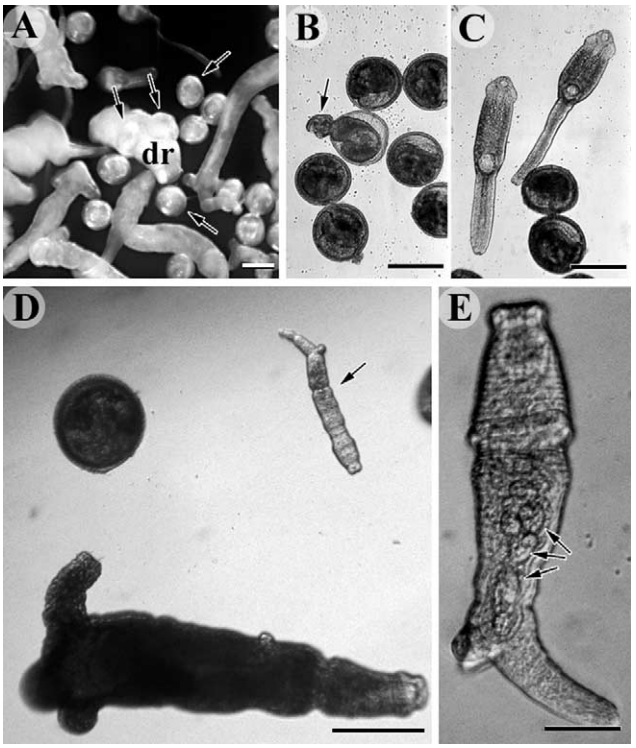

Fig. 3. Events observed during cultivation. (A) Encysted metacercariae (arrows) derived from cercariae released in vitro, dr—dead redia; (B) marita (arrow) excysting from metacercaria; (C) juvenile maritae; (D) progeny redia (arrow) released at the 90th day of cultivation; scale bar = 250 μm (A–D); (E) progeny redia containing germinal balls (arrows), scale bar = 50 μm.

active and began to turn and twist under cyst envelopes in all directions. Such larvae activity resulted in the excystment of juvenile maritae (Figs. 3B and C) which were weakly motile in the beginning and dead within 3–4 days. To note, if warmed up to 40–42 °C, maritae vigorous activated and showed specific body bending. Attached to the substrate with both suckers, they lifted posterior body part almost perpendicular to the plastic surface.

Generally, short-term cultures with rediae derived from naturally infected periwinkles contained only daughter rediae releasing cercariae. However, one redial "clone" in the long-term cultivation experiment at day 90 postculture initiation was found to release several progeny rediae producing germinal material (Figs. 3D and E). Though these young rediae initially were very active, they did not grow, their germinal balls did not increase in size and they died within 3 weeks.

When pulsed with ^{14}C-labeled amino acids mixture, rediae showed evident signs of high metabolic activity. About 25 proteins were newly synthesized in the course of 24 h in detectable quantity (Fig. 4). Important to note is the absence of any differences in protein compositions and protein synthesis between the first and the sixth day of cultivation.

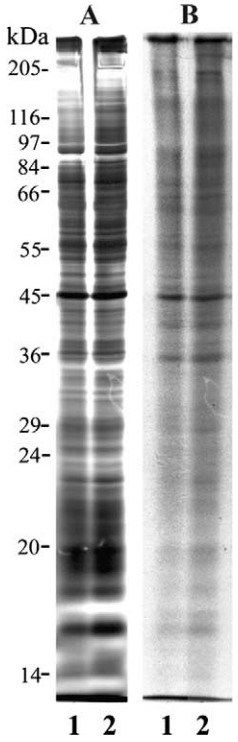

Fig. 4. Protein profiles of *H. elongata* rediae (A) and rediae total protein synthesis (B) at day 1 (1) and 6 (2) of the culture period. Rediae cultures were pulsed for 24 h at day 1 and 6 with ^{14}C–amino acids mixture (100 μCi/ml). (A) Silver staining of reducing SDS–PAGE-separated proteins (12% gel); (B) fluorograms of rediae ^{14}C-labeled proteins.

Means of total proteolytic activity accumulated in culture medium for 48 h in 4- and 6-days cultures ($A_{490} = 0.015$ and 0.013 accordingly) were near by detection level (for several enzymes is about $A_{490} = 0.02$ according to the PADK protocol) and did not differ significantly ($P > 0.05$). Both estimates were significantly lower ($P \ll 0.0001$) than that of positive control ($A_{490} = 1.01$).

4. Discussion

In our culture system moribund or dead cercariae, their lost tails and dead rediae bodies were the only protein source for the rediae. Similar propensities for scavenging and predating were shown in *Echinostoma caproni* rediae (Loker et al., 1999) and seem natural for Echinostomatidae, histiophagy traditionally attributed to them. In this study, consuming of dead neighbors supplied rediae with energy enough for continued survival (more than 5 months) and development of in vivo preformed progeny, but not for forming new embryos. The only exclusion is progeny rediae, which were released after 3 months of cultivation with already formed germinal balls. Basch and DiConza (1975) showed that *Echinostoma paraensei* rediae dissected from *Biomphalaria glabrata* at the 18th day postexposure (dpe) to a miracidium released progeny rediae in axenic culture. In synxenic culture, *E. caproni* individual mother rediae derived from the snail at 20 dpe produced daughters ingesting Bge cells and in some cases containing developing germinal material (Loker et al., 1999).

As all the rediae placed in the culture were obtained from naturally infected snails, it is difficult if at all possible to identify their individual age and general age of redial microhemipopulation. In the White Sea *L. littorea* survive for up to 32 yr and, as shown in mark-recapture experiments, individuals infected with *H. elongata* can support the established parasite with certainty for at least 2 yr (own unpublished data). It seems likely that the age of redial microhemipopulation obtained from snails in our experiments might vary within this time range. Despite the fact that individual age of dissected rediae is uncertain and a priori cultivation in vitro poorly reflects natural situation, the data obtained in long-term survival experiment clearly show that individual longevity potential of *H. elongata* rediae is more than 5 months. To note, the life span of rediae of related echinostome species infecting *B. glabrata* is much shorter in similar axenic cultures—20 days for *E. caproni* (Loker et al., 1999) and about a month for *E. paraensei* (Basch and DiConza, 1975). Keeping in mind sharp distinction between life spans of *L. littorea* (about 30 yr) and *B. glabrata* (annual), the life span of echinostome rediae may correlate with that of the host.

Encystment of cercariae after continuous swimming seems to contradict the data on other echinostome cercariae studied up to now. Both of the previously studied species, *E. paraensei* (Stein and Basch, 1977) and *E. caproni* (Loker et al., 1999) were capable of normal encystment in cultures containing Bge cells but not in unconditioned medium. In our parallel studies we observed spontaneous encystment (less than 1%) of 24-h-old *H. elongata* larvae in pure seawater and triggering effect of some non-specific proteins, e.g., bovine serum albumin (BSA), which significantly increase encystment rate in the end of the larvae life. It is quite possible that if an elderly cercaria has the only alternative—to encyst or to die—the presence of secretory–excretory proteins is the factor needed for the choice of the first option.

The appearance of juvenile maritae in this study was unexpected as metacercariae of *H. elongata* should excyst in the seagull intestine under much higher temperature and, if we use analogy with other echinostome species (reviewed in Fried, 1994), in the presence of sufficient quantities of proteolytic enzymes. In principle, the conditions of employed culture favor the accumulation of proteases originating from dying tissue and cells, released from well-developed gut of rediae and penetration glands of cercariae. However, in our culture system with relatively often changed medium, no accumulation of proteolytic activity could be detected with confidence.

Irwin et al. (1984) treated *Himasthla leptosoma* metacercariae in a medium containing sodium taurocholate, trypsin and 0.8% (w/v) L-cysteine at 41 °C. This treatment resulted in intense metacercarial activity and emergence of larvae through the cyst wall. Interestingly, L-cysteine is a part of L-15 medium we used, however its concentration was 60 times lower than in the experiments by Irwin et al. (1984). Whether the excystment of juveniles is due to mechanical or biochemical factors or the result of in vitro artifact remains to be determined. In any case, the possibility to obtain aseptically the youth of hermaphroditic generation gives a good chance for further development of appropriate culture system allowing *H. elongata* maritae to oviposit in vitro.

No attempt was made to compare various kinds of media in this study. However, many researches have suggested that L-15 medium is the best choice for propagation of marine invertebrate cells, due to its strong buffering capacity and its chemical composition (reviewed in Odintsova, 2001). This medium was favorable to *H. elongata* rediae during all stages of cultivation and we did not note any visible indications of initially stressful situation requiring adaptation period like it was noted for *E. caproni* rediae in the presence of Bge medium (Loker et al., 1999). Our preliminary studies showed great potential of L-15 medium for primary cultures of *L. littorea* and *M. edulis* hemocytes. They survive and demonstrate some cell reactions adequately for up to one month. Having in view uniform protein synthesis in the course of at least the first week of rediae cultivation and minute proteolytic activity of redia-conditioned medium, the culture system suggested in this study is a valuable tool permitting us not only to characterize the soluble factors the parasite secretes, but also to investigate interference of these factors with host cells.

Acknowledgments

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