# *Biomphalaria glabrata* embryonic (Bge) cell line supports *in vitro* miracidial transformation and early larval development of the deer liver fluke, *Fascioloides magna*

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SUMMARY

A Bge cell co-culture system, previously shown to support the *in vitro* production of daughter sporocysts from mother sporocysts of *Schistosoma mansoni* and *S. japonicum*, has proven capable of supporting the *in vitro* development of intramolluscan stages of the deer liver fluke, *Fascioloides magna*. Miracidia commenced transforming within 4 h of incubation with Bge cells, and had completely shed their epidermal plates within 18–24 h. Redial stages were visible inside *in vitro*-transformed mother sporocysts after 12–16 days of co-culture with Bge cells, and emerged as fully-developed larvae starting at 14–20 days post-cultivation. Rediae survived over 60 days of *in vitro* culture, and reached a maximum size of 150–170 $\mu$m. Although particulate material was visible in their cecae, rediae were not observed to actively feed on Bge cells. Bge cells did not attach to or encapsulate larval stages at any time throughout the incubation period. Unlike *Schistosoma* spp., in which a high percentage of miracidia spontaneously shed their ciliated epidermal plates and transformed into mother sporocysts in Chernin's balanced saline solution (CBSS), transformation of *F. magna* was dependent on Bge cell products. Less than 5 % of *F. magna* miracidia transformed spontaneously in either CBSS or Bge medium with 10 % fetal bovine serum (complete or C-Bge). However, incubation of miracidia in either Bge cell-conditioned C-Bge medium or a greater than 30 kDa fraction concentrated from conditioned CBSS increased transformation rates to 67 and 83 %, respectively. This secretory Bge cell factor(s) appeared to be protein in nature since its activity was completely abrogated by heat or proteinase K treatments. Overall, these results demonstrate that Bge cells are required for stimulating *in vitro* miracidial transformation and supporting early larval development of a fasciolid trematode under culture conditions. This is the first report of *in vitro* development of rediae from miracidia for a digenetic trematode.

Key words: *Fascioloides magna*, deer liver fluke, *Biomphalaria glabrata* embryonic (Bge) cell line, *in vitro* cultivation, miracidial transformation, mother sporocyst, redia.

## INTRODUCTION

The life-history of virtually all digenetic trematodes, or flukes, requires development in snail intermediate hosts. This intramolluscan development includes extensive asexual multiplication and progression through a series of larval sporocyst and/or redial generations. Although the life-cycles of numerous trematodes have been described (Schell, 1985), even in well-studied parasites such as *Schistosoma* spp. (Cheng & Bier, 1972; Meuleman, 1972; Schutte, 1974; Meuleman, Holzmann & Peet, 1980; Theron & Touassem, 1989) and *Fasciola hepatica* (Kendall, 1965; Wilson, Pullin & Denison, 1971; Wilson & Draskau, 1976; Koie, Christensen & Nansen, 1976), little is known about the chemical or molecular cues important in signalling transformation of the miracidium to its successive larval stages or factors responsible for subsequent larval development. Donges & Gotzelmann (1988) reported that intramolluscan asexual replication of some digeneans is potentially unlimited, but the stimuli important for asexual multiplication during this complex larval development are largely unknown.

In addition to the complexity of larval development, most fluke species exhibit high specificity for their gastropod intermediate hosts. This strict specificity can be seen, not only at the interspecific level, but also as intraspecific strain-level differences in susceptibility between snails from different geographic regions (Basch, 1976) or between lab-derived isolates (Adema & Loker, 1997). Although the resistance to infection has been shown to be mediated, to a large extent, by haemocyte-mediated killing (van der Knapp & Loker, 1990; Bayne & Yoshino, 1989; Yoshino & Vasta, 1996; Adema & Loker, 1997), the molecular mechanisms underlying these cellular responses are still poorly understood. In fact, the ability of a given strain of parasite to infect a particular snail strain may depend on the availability of necessary components (e.g. essential

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nutrients or developmental cues) in susceptible strains that are missing in resistant strains, as well as the inability of susceptible host strains to mount effective immune reactions.

*In vitro* cultivation of trematode larval stages provides a potentially valuable tool for studying the molecular interactions important in trematode development and determining molecular mechanisms underlying snail susceptibility. Successful cultivation from miracidium to daughter sporocysts of both *S. mansoni* (Yoshino & Laursen, 1995) and *S. japonicum* (Coustau *et al*. 1997), as well as development of mother sporocysts of *Echinostoma caproni* (Ataev, Fournier & Coustau, 1998) in co-culture with a *Biomphalaria glabrata* embryonic (Bge) cell line have recently been reported. *S. mansoni* and *E. caproni* typically use *B. glabrata* as an intermediate host, and, although *S. japonicum* uses a distantly related snail species (*Oncomelania* spp.), all of these systems used lab-adapted strains of parasites.

In this study we report the capacity of the Bge co-culture system to support transformation and larval development of wild caught miracidia of the deer liver fluke, *Fascioloides magna*. This trematode typically uses lymnaeid snails as an intermediate host, and unlike the lab-adapted schistosomes, miracidia of *F. magna* do not spontaneously transform into sporocysts under *in vitro* conditions in the absence of snails. This is the first demonstration of snail cell-derived factors important in *in vitro* miracidial transformation to sporocysts, and the first report of sequential development of miracidia through mother sporocysts to mother rediae under culture conditions.

## MATERIALS AND METHODS

### *Parasite isolation and* in vitro *culture conditions*

Adult *Fascioloides magna* were recovered from hunter-killed deer in St Croix State Park in Minnesota in November of 1996. Deer livers were sliced at approximately 2 cm intervals to reveal encysted adult flukes, which were removed from opened cysts and placed into warm saline for approximately 2 h. Fluid in the cysts, composed of fluke vomitus, sloughed host tissue, and free trematode eggs, was collected by pipette. Eggs shed into the saline and those in cyst fluid were separately washed and concentrated by repeated sedimentation in water. Cleaned eggs were then allowed to embryonate at 26 °C, for 2–3 weeks, in deionized water containing activated charcoal until miracidia were visible and active in the eggs. Embryonated eggs were then stored in the same water at 4 °C until used. Under these conditions, eggs remained viable for more than 1 year. All eggs used in our experiments, conducted from July 1997 through February 1998, came from the same stock. Miracidia were hatched from embryonated eggs by warming them to 26 °C for 15 min. Swimming miracidia were then collected by Pasteur pipette and transferred to sterile 15 ml tubes containing sterile pond water containing penicillin G (100 U/ml: Sigma Chemical Co., St Louis, MO), and streptomycin sulfate (0·05 mg/ml: Sigma). Tubes were wrapped to within 1 cm of the top with aluminum foil and illuminated with a lucite lamp source. Miracidia, concentrated at the lighted top of the tube, were collected and transferred to a new sterile tube. This procedure was repeated 5 times. After the final wash in water, miracidia were washed twice in Chernin's balanced salt solution (CBSS: Chernin, 1963) before use.

### *Establishment of* F. magna *– Bge cell co-cultures and transformation assays*

For co-culture experiments, *F. magna* miracidia in CBSS were added to 30 ml tissue culture flasks containing established monolayers of *B. glabrata* embryonic (Bge) cells in Bge medium (Hansen, 1976) supplemented with 10 % heat-inactivated fetal bovine serum (complete or C-Bge medium). Bge cells were originally obtained from American Type Culture Collection (ATCC CRL 1494; Rockville, MD).

Transformation assays were conducted in 96-well tissue culture plates. Each well contained 200 $\mu$l, made up of equal volumes of media to be tested and miracidia in CBSS. Test media included: CBSS; conditioned CBSS (CBSS recovered from overnight incubation with previously established Bge cell monolayers); > 30 kDa conditioned-CBSS (conditioned CBSS retained by a 30 kDa cutoff ultrafiltration membrane; Amicon Inc., Beverly, MA); C-Bge medium; conditioned C-Bge medium (C-Bge medium recovered from Bge cell cultures); or C-Bge medium plus Bge cells (co-culture). Modification of the above assays included heat-treatment (56 °C for 30 min, or 100 °C for 10 min), or proteinase treatment (1 U proteinase K-agarose (Sigma) for 30 min at 22 °C). All assays were conducted at 26 °C under normal atmospheric conditions. Assay cultures were observed at 2, 4 and 18 h, and thereafter daily, using a Nikon Diaphot inverted microscope equipped with an N600G 35 mm camera. Miracidia were scored as either untransformed (swimming miracidia and transforming miracidia settled to the bottom with ciliated epidermal plates rounded up but still attached) or transformed sporocyst (loss of epidermal plates). Statistical analysis was done using Instat software (Ver.2.06, Graph Pad Software).

## RESULTS

The series of events that characterized *in vitro* transformation of miracidium to mother sporocyst in *F. magna* were as follows: Miracidia (Fig. 1 A) swam

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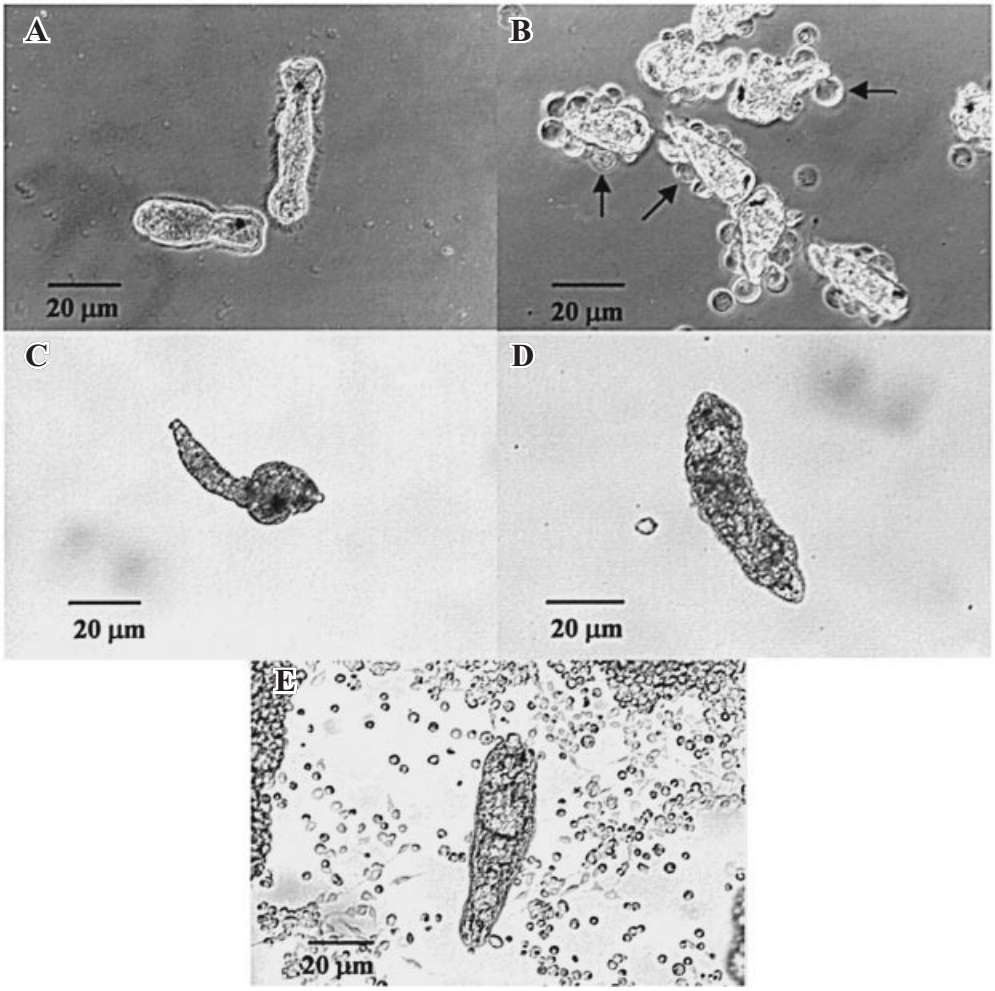


Fig. 1. Comparison of *in vitro* transformation of *Fascioloides magna* miracidia and sporocyst development under various culture conditions. (A) *F. magna* miracidia after 12 h in CBSS at 26 °C. (B) *F. magna* miracida following 12 h of incubation in *Biomphalaria glabrata* embryonic (Bge) cell-conditioned CBSS at 26 °C. Ciliated plates being shed by sporocysts are indicated by arrows. (C) Mother sporocyst of *F. magna* cultured for 10 days at 26 °C in fresh, complete Bge cell (C-Bge) medium. (D) *F. magna* mother sporocyst cultured for 10 days at 26 °C in Bge cell-conditioned C-Bge medium. (E) *F. magna* sporocyst co-cultured with Bge cells in C-Bge medium for 10 days at 26 °C.

in straight-line patterns, spiralling through the medium. Transforming stages eventually (within 3–6 h) settled to the bottom of the well, and their ciliated epidermal plates, which had been tightly adherent at the miracidial surface, began rounding up and detaching from the larval surface (Fig. 1 B). Cilia on detached rounded plates continued to beat for a short period, but eventually plates would lose their cilia and lyse. Fully transformed mother sporocysts, depicted in Fig. 1 D and E, lacked ciliated epidermal plates, and were capable of only limited motility by whole body contractions. However, flame cell activity was clearly visible in live sporocysts. Eyespots and prominent apical papilla also were retained in sporocysts following transformation.

Bge cells, or secreted Bge cell products, were required to stimulate transformation of miracidia (Fig. 2). One-way ANOVA showed that there was a significant difference between the various treatment groups in the percentage of transformed sporocysts produced in either CBSS-based wells ($F = 35{\cdot}240$, $P < 0{\cdot}0001$) or C-Bge medium-based wells ($F = 77{\cdot}297$, $P < 0{\cdot}0001$). Only 5 % and 1·5 % of miracidia transformed within 24 h in either control C-Bge medium or control CBSS, respectively. The addition of equal volumes of conditioned C-Bge medium, conditioned CBSS, or $> 30$ kDa fraction of conditioned CBSS increased the transformation rate to 66·7, 83·7 and 82·7 %, respectively, which was significantly higher than control wells ($P < 0{\cdot}0001$). Both heating (56 °C for 30 min, or 100 °C for 10 min) and proteinase K treatment of conditioned C-Bge or conditioned CBSS significantly reduced transformation rates ($P < 0{\cdot}0001$). There was no significant difference between control wells and heat-treated wells in C-Bge medium or CBSS. The transformation rate in proteinase K-treated conditioned CBSS wells remained significantly higher than control CBSS wells, but proteinase K-treated conditioned C-Bge was not significantly above control C-Bge medium.

Miracidia began transforming within 4 h of in-

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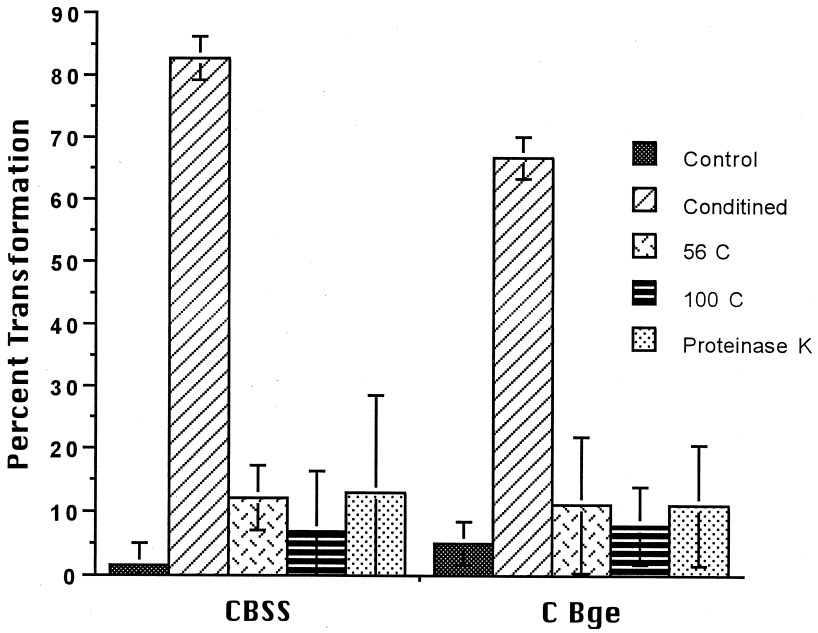


Fig. 2. Percentage (mean ± S.D.) of *Fascioloides magna* miracidia that transformed into mother sporocysts in either CBSS or C-Bge medium. Media treatments included fresh control, Bge cell-conditioned, 56 °C for 30 min, 100 °C for 10 min, and proteinase K-treated.

cubation in wells containing conditioned CBSS, a > 30 kDa fraction of conditioned CBSS, or conditioned C-Bge media, and were fully transformed within 18–24 h. Viability of sporocysts, previously transformed in conditioned Bge medium and then transferred to complete Bge media, conditioned Bge media, or Bge cell co-cultures, varied between medium treatments. By 10 days post-transformation, the vast majority of sporocysts cultured in fresh C-Bge medium alone were dead. Those few still living were shrunken, and exhibited abnormal growth (Fig. 1 C). Approximately 50 % of sporocysts transformed and incubated in conditioned C-Bge medium remained alive after 10 days in culture (Fig. 1 D), while most (> 90 %) of sporocysts co-cultured with Bge cells survived during this same culture period (Fig. 1 E).

Sporocysts, transformed and maintained in co-culture with Bge cell monolayers, went on to undergo further larval development. After 2–4 days of culture, transformed sporocysts were devoid of ciliated epidermal plates and each possessed what appeared to be a primordial embryonic mass or dense cell aggregate (Fig. 3 A). With continued cultivation, these embryonic cell masses/aggregates continued to enlarge within some sporocysts (Fig. 3 B) and, by 12–16 days of cultivation, independently motile rediae were recognized within these sporocysts (Fig. 3 C). Starting between days 14 and 20 post-cultivation, fully-developed rediae were observed being expelled from sporocysts by way of a birth pore at the posterior end (Fig. 3 D). Only about 1 % of cultured sporocysts were capable of producing redial stages under various *in vitro* conditions, and invariably, only a single redia was produced by each. Newly-released rediae were easily distinguished from sporocysts by the presence of a mouth with associated pharynx, and ambulatory buds (Fig. 1 E). These rediae continued to grow, obtaining a maximum length of over 160–170 $\mu$m, and remained viable for > 60 days in culture (Fig. 3 F), although no further development of successive embryonic stages was observed. Sporocyst and redial stages remained motile and free of adhering Bge cells throughout the incubation. Although particulate material was occasionally visible in their cecae, rediae were not observed to actively feed on Bge cells or cell aggregates.

## DISCUSSION

The Bge cell line, derived from *B. glabrata*, was first shown to be capable of supporting *in vitro* development of intramolluscan stages of *S. mansoni* through production of daughter sporocysts (Yoshino & Laursen, 1995). Since then the system also has been found to support similar *in vitro* development of *S. japonicum* (Coustau *et al.* 1997). Both of these schistosome species undergo similar larval development which includes daughter sporocysts giving rise to cercariae, but *S. mansoni* uses *Biomphalaria* spp. as natural intermediate hosts, while *S. japonicum* uses *Oncomelania*, a distantly related species of the snail. The ability of Bge cells to support development of *S. japonicum* sporocyst development was surprising in light of the high degree of snail host specificity normally exhibited by larval schistosomes (Basch, 1976). Since *F. magna* typically uses lymnaeid snails as intermediate hosts, our present finding that Bge cells support development of *F.*

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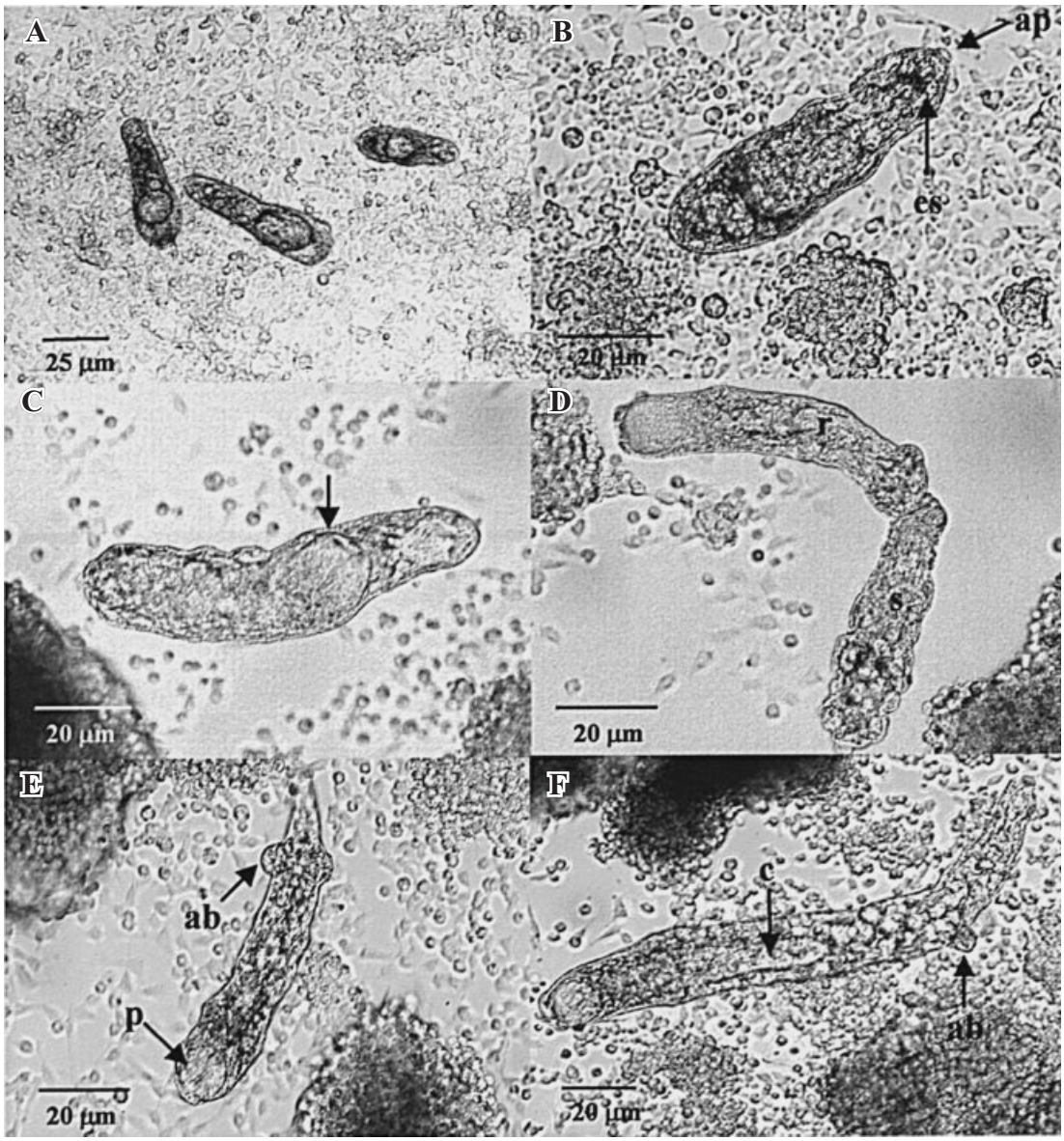


Fig. 3. *In vitro* development of larval *Fascioloides magna* maintained in co-culture with *Biomphalaria glabrata* embryonic (Bge) cells. (A) *In vitro* transformed *F. magna* mother sporocysts after 4 days in culture. (B) 10-day-old mother sporocyst with developing embryo. Note that sporocyst still retains eyespot (es) and apical papilla (ap) at anterior end. (C) 16-day *F. magna* sporocyst containing fully-differentiated redia. Arrow denotes muscular pharynx with oral opening. (D) Redia (r) newly-emerging from posterior end of *F. magna* mother sporocyst (s) at 20-days post cultivation. (E) Redial stage, 25 days after initial larval cultivation. ab, ambulatory bud; p, pharynx. (F) Redia in 45-day-old culture. ab, ambulatory bud, c, cecum.

*magna* miracidia through the mother redial stage under *in vitro* conditions, provides further evidence for the possible existence of a developmental factor(s) provided by Bge cells that is shared in common by diverse trematode species. There is one report of a naturally acquired infection of *B. alexandrina* by *Fasciola gigantica* (Farag & el Sayad, 1995). The apparent ability of some fasciolids to infect *Biomphalaria* spp. may help explain the ability of *F. magna* to develop in the presence of Bge cells. On the other hand, the ability to use *B. glabrata* as a compatible intermediate host does not guarantee successful *in vitro* development. Ataev *et al*. (1998) recently used the Bge cell co-cultivation system with mother sporocysts of *Echinostoma caproni*, which also uses *B. glabrata* as a suitable snail host, but were unable to generate either secondary sporocysts or rediae. This result was unexpected because *E. caproni*, and echinostomes in general, use multiple snail hosts in their life-cycle so they may already be predisposed to tolerate a much wider spectrum of physiological environments in their development. Thus, the nature of the *in vitro* molecular signals being provided by this cell line and responded to by different trematode species is complex, and their identification ultimately may be important in elucidating the molecular basis for host specificity in digenetic trematodes.

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Miracidia of *S. mansoni* are known to undergo spontaneous transformation into mother sporocysts when cultivated in saline (Targett & Robinson, 1964; Samuelson, Quinn & Caulfield, 1984), CBSS (Conners & Yoshino, 1990), or C-Bge medium (Basch & DiConza, 1974; Lodes & Yoshino, 1989). This transformation was independent of snail-derived factors, but also occurred in the presence of Bge cells (Yoshino & Laursen, 1995). *In vitro*-derived mother sporocysts, however, were unable to progress through larval development in the absence of Bge cells. Similar miracidial transformation patterns have been observed for *S. japonicum* (Coustau *et al*. 1997) and *Echinostoma* spp. (Adema *et al*. 1994).

In contrast to other digenean species, we have shown that *F. magna* miracidia were unable to transform spontaneously into mother sporocysts in simple saline or complex medium alone, but did so in the presence of co-cultured Bge cells, suggesting some fundamental difference in the transformation process of *F. magna* miracidia. Moreover, *F. magna* transformation did not require direct cell contact, since miracidia transformed in conditioned media, presumably containing only Bge cell-derived products. *F. magna* has been reported to use several species of lymnaeid snails as intermediate host (Krull, 1934; Foreyt & Todd, 1978; Griffiths, 1959, 1973). Typically, miracidia of this species attached via their apical cone and shed their cilia either on the outer surface of the snail or as they penetrated into the snail, but epidermal plates were often carried into the snail (Coil, 1977, 1981). There has been 1 report of transformation of *F. magna* miracidia into sporocysts *in vitro* (Campbell & Todd, 1955), but only after miracidia, which had initially attached to a snail, then detached and subsequently continued to shed epidermal plates and transform into mother sporocysts off the snail.

The molecular signal(s) responsible for triggering the shedding of ciliated plates following snail contact (Campbell & Todd, 1955) or Bge cell cultivation (this study) are unknown. However, based on membrane ultrafiltration, heat-treatment, and proteinase K treatment, the Bge transformation factor(s) appears to be a protein(s) with a molecular weight of $> 30$ kDa. Although it may be that this transformation factor was a breakdown product of some serum protein component of the medium, the extensive washing of Bge cells prior to culturing in CBSS during the preparation of conditioned-CBSS makes this an unlikely possibility. Instead, we believe that this protein was synthesized and secreted directly by Bge cells. We are currently investigating this hypothesis.

The miracidial transformation signal was insufficient for further larval development or differentiation, because, in the absence of Bge cells or their products, most mother sporocysts died within 10–12 days of incubation without developing germ balls or rediae. By contrast, in the presence of Bge cells, mother sporocysts continued to grow and a small percentage went on to produce redial stages as early as 14 days post-transformation. However, like *S. mansoni* (Yoshino & Laursen, 1995) and *S. japonicum* (Coustau *et al*. 1997), culture conditions were far from optimal as indicated by a severely reduced reproductive capacity. Only a very small percentage ($< 1\,\%$) of sporocysts produced rediae and only 1 redia per sporocyst was observed. After rediae emerged, they were quite active and continued to grow for at least 60 days in culture. However, again, mother rediae died without any further embryo development or producing daughter rediae. The inability to sustain larval development may have been due to various factors including insufficient or improper stimuli for triggering differentiation of the next larval generation, lack of proper nutrition, buildup of metabolic waste products during the extended incubation period or a combination of these.

Similar to mother sporocysts of *S. japonicum* (Coustau *et al*. 1997) and *E. caproni* (Ataev *et al*. 1998), Bge cells did not adhere to or encapsulate developing *F. magna* sporocysts, nor did larvae burrow into cell aggregates. This was quite different than co-cultures of *S. mansoni* in which Bge cells completely encapsulated the parasites without killing them (Yoshino & Laursen, 1995). We interpret this more intimate interaction as being related to host specificity. Since *S. mansoni* uses *B. glabrata* as one of its natural intermediate hosts, it is possible that specific surface molecules necessary for the binding of Bge cells to parasites were compatible in the *S. mansoni* system, but not with trematodes that do not normally use *B. glabrata* as a host. Although *E. caproni* also can use *B. glabrata* as a compatible host, the fact that rediae were not produced in Bge co-cultures suggests that the molecular relationship for sustaining advanced MS development was sub-optimum. Perhaps the inability of Bge cells to adhere to *E. caproni* sporocysts is a reflection in this *in vitro* molecular imbalance. Snail cell interaction with sporocysts varies even between snail strains. Haemocytes from a susceptible (PR alb) *B. glabrata* strain adhered to *S. mansoni* sporocysts but did not kill them, while haemocytes from a resistant (10R2) strain both encapsulated and killed sporocysts, probably aided by unidentified plasma-borne factors (Bayne, Buckley & DeWan, 1980). The specific molecules important for sporocyst recognition, binding, and killing by haemocytes are still unknown.

In summary, this is the first report of advanced *in vitro* larval development in a fasciolid trematode. In this case, developmental signal(s) provided by cells of the Bge cell line appear to be appropriate since *F. magna* mother sporocysts gave rise to rediae, the next

larval stage normally produced under *in vivo* conditions. The ability of Bge cells to support larval differentiation and growth of trematode species that do not ordinarily use *B. glabrata* as a natural intermediate host is significant in that it now permits the identification and study of non-specific factors potentially important in signalling miracidial transformation and early intramolluscan development. It is hoped that the recent reports of transfection systems based on Bge cells (Lardens *et al*. 1996; Yoshino, Wu & Liu, 1998) may make it possible to study the effect of specific gene products on larval trematode development and differentiation. In addition, the differential ability of Bge cells to adhere to various, even closely related, trematode species may make it possible to use this co-cultivation system to determine important molecular-level interactions in molluscan defence mechanisms.

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