IN VIVO AND ECTOPIC ECYSTATION OF ECHINOSTOMA REVOLUTUM AND CHEMICAL ECYSTATION OF THE METACERCARIAE

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ABSTRACT: In vivo and ectopic encystment of the cercariae of Echinostoma revolutum from Lymnaea elodes snails in Indiana and chemical encystation of the metacercariae were studied. In vivo encystment occurred in adults of Biomphalaria glabrata and Helisoma trivolvis (Colorado strain) snails and in neonatal and juvenile L. elodes snails. These results were expected because 37-collar-spined Echinostoma species show broad specificity in their second intermediate gastropod hosts. Encysted metacercariae of E. revolutum and Echinostoma trivolvis removed from experimentally infected snails and treated in a trypsin–bile salts excystation medium at 39 C showed 30.3% excystation for the former and 55.7% for the latter at 4 hr. The ducts and openings of the paracloacal glands of encysted metacercariae of E. revolutum from cysts formed in snails did not stain with neutral red. Abnormal ectopic cysts with distorted outer walls and granular inner walls were obtained within 48 hr of placing E. revolutum cercariae in Locke’s 1:1 plus 1% dextrose. These metacercariae encysted rapidly in the excystation medium and their paracloacal gland ducts and openings stained with neutral red. Differences in ectopic encystment and chemical excystation in vitro can be used to distinguish these closely related species in the E. revolutum complex.

Sorensen et al. (1997) described a strain of Echinostoma revolutum from Lymnaea elodes snails in Indiana. Cercariae released from the limnaeids encysted in various pulmonate snails and the cysts when fed to domestic chicks yielded ovigerous adults. This was the first unequivocal report of E. revolutum in North America. The identity, systematic status, and biology of the larval and adult stages of E. revolutum were reviewed by Kanev (1994). Experimental studies on larvae and adults of E. revolutum are sparse compared to those on Echinostoma trivolvis, a related 37-collar-spined species from Helisoma trivolvis snails in North America. The identity of this species and a redescriptions of larval and adult stages have been reported by Kanev et al. (1995). The E. trivolvis–H. trivolvis model has been used extensively in the U.S.A. (see Huffman and Fried, 1990, for review) and information is available on cercarial longevity (Pechnik and Fried, 1995), cercarial encystment in pulmonate snails (Anderson and Fried, 1987; Fried et al., 1995; Schmidt and Fried, 1996a), on chemical excystation of the metacercariae (Smoluk and Fried, 1994), and on ectopic encystment of the cercariae (Fried and Bennett, 1979); however, similar studies on E. revolutum are not available. To increase our knowledge of the biology of 37-collar-spined echinostomes in the E. revolutum complex, studies on in vivo and ectopic encystment and chemical excystation of the metacercariae of E. revolutum were done and compared with those on E. trivolvis.

MATERIALS AND METHODS

Cercariae of E. revolutum were obtained from 8 experimentally infected L. elodes snails, 20–30 mm in shell length. Each snail was isolated in a Stender dish with 5 ml of artificial spring water (ASW) as described in Schmidt and Fried (1996b). From 100 to 500 cercariae per snail were obtained within 4 hr. Cercariae were pooled from at least 2 snails and used within 4 hr postemission.

To obtain in vivo encysted metacercariae, 10 Biomphalaria glabrata and 20 H. trivolvis (Colorado [CO] strain) (both labared and 6–8 mm in shell diameter) were each exposed to 25 cercariae in multicell chambers (Schmidt and Fried, 1996a), necropsied 24 hr later, and the cysts were counted. In 1 experiment, 10 lab-reared juvenile L. elodes snails, 1–2 mm in shell length, were each exposed to 25 cercariae. In another experiment (en masse exposure), about 25 newly hatched (neonatal) L. elodes, 1 ± 0.2 mm in shell length, were maintained for 2 days in 800 ml of ASW with 2 adult L. elodes that were emitting cercariae. Cercarial encystment in a representative sample of 10 of the newly hatched limnaeids was determined on the second day.

Cercarial longevity studies were done in ASW or ASW with 1% dextrose at 22–24 C and 4 C. Longevity was based on cercarial movement or response to a needle. The infectivity of aged cercariae was tested by placing them at room temperature in multiwell chambers containing 1.5 ml of ASW with H. trivolvis (CO strain, 5 mm in shell diameter). Three snails per solution were exposed individually to 25 cercariae and necropsied 24 hr postinfection (PI). Ectopic encystment on 25 E. revolutum cercariae/dish was studied in 6-cm diameter petri dishes containing 15 ml of either Locke’s, Locke’s plus 1% dextrose, 1:1 Locke’s, 1:1 Locke’s plus 1% dextrose, ASW, or ASW plus 1% dextrose. Ectopic encystment was also tested in the presence of mucus from B. glabrata, H. trivolvis (Pennsylvania and CO strains), and L. elodes. Mucus was collected by placing 2 snails of each species in 0.5 ml of ASW in a 6-cm diameter petri dish for 1 hr.

To examine excystation of ectopic cysts, 75 were treated in the chemical excystation medium of Fried and Roth (1974) as described below.

To examine chemical excystation of cysts formed in vivo, a total of 300 encysted metacercariae of E. revolutum was treated in the alkaline trypsin–bile salts medium of Fried and Roth (1974) at 39 C and similar experiments were done with E. trivolvis cysts. In the present study, cysts of both species were used within 2 wk of cercarial encystment in experimentally infected H. trivolvis (CO) snails. In each of 3 trials, 100 E. revolutum and 100 E. trivolvis cysts were placed in 4 ml of the medium, incubated at 39 C for 4 hr, and the number of activated, breached, and encysted metacercariae was determined.

RESULTS

Studies on 100 cercariae per experiment in ASW at 22–24 C showed that most cercariae were sluggish after 12 hr, but survived for 22 hr in ASW and 26 hr in ASW plus 1% dextrose. However, aged cercariae (more than 12 hr old) could not infect lab-reared H. trivolvis (CO) snails, as none of the snails exposed to these cercariae contained encysted metacercariae. Cercariae maintained for 24 hr in ASW at 4 C, then transferred to ASW at 22–24 C for 1 hr were active and the mean number ± SE of these cercariae recovered as cysts in H. trivolvis (CO) was 9.3 ± 0.9 per snail at 24 hr PI. However, cercariae maintained at 4 C in ASW plus 1% dextrose were sluggish when transferred to ASW at 22–24 C for 1 hr, and only 3.3 ± 0.9 (mean ± SE) of these cercariae were recovered as cysts in H. trivolvis (CO) at 24 hr PI.

Ectopic encystment rarely occurred in Locke’s 1:1 at 22–24

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C within 48 hr and never occurred in Locke’s, ASW, or ASW plus 1% glucose. Ectopic encystment did not occur on the mucus trails of H. trivolvis, B. glabrata, or L. elodes. Results of ectopic encystment in Locke’s 1:1 plus 1% glucose in 8 separate trials after 48 hr showed that in 6 trials the number of ectopic cysts ranged from 10 to 22 (average 17.5). Ectopic encystment did not occur in 2 trials. The mean ± SE of ectopic cysts from all trials was 13.1 ± 3.2. Ectopic cysts had grossly distorted outer cysts and poorly formed granular inner cysts (Fig. 1). About one-half of the ectopic cysts retained their cercarial tails (see Fig. 1). Cercariae that did not encyst within 48 hr were dead or moribund.

Of 75 ectopic cysts treated in the excystation medium, 20 were breached (Fig. 2) and 20 were excysted (Figs. 3, 4) after 1 hr. Ten excysted metacercariae stained in 0.01% neutral red showed staining in the ducts (Fig. 3) and openings (Fig. 4) of the paraesophageal glands. Echinostoma revolutum cercariae have numerous paraesophageal glands with ducts and openings that stain with 0.01% neutral red (see photomicrograph in Fig. 5 of this paper and line drawing in Fig. 10 in Kanev, 1994).

The mean ± SE number of E. revolutum cysts recovered from the H. trivolvis (CO) snails was 11.2 ± 0.9 (range 4–16) and 10.1 ± 1.5 (range 4–20) from the B. glabrata snails.

In the experiment in which newly hatched L. elodes were exposed en masse to E. revolutum cercariae, all 10 snails were infected with a mean ± SE of 9.8 ± 1.2 cysts (range 2–13 cysts). Of 50 cysts (Fig. 6) treated in the excystation medium immediately after snail necropsy, 25 were either breached (Fig. 7) or excysted (Fig. 8) within 2 hr. There was no neutral red staining of the paraesophageal glands of 10 excysted metacercariae (see Fig. 8).

In the experiment in which 10 juvenile L. elodes were exposed individually to 25 cercariae and necropsied 24 hr later, a total of 121 cysts were recovered (range 6–24 per snail, mean ± SE per snail = 12.1 ± 1.8). Results of excystation experiments and observations on excysted metacercariae stained in neutral red were similar to those described for en masse infections.

Comparative studies on chemical excystation of E. revolutum and E. trivolvis cysts from snails were similar, revealing activation or rotation of the larva within the cyst, followed by breaching (Fig. 7) of the inner cyst via a specific result in a larva that stretched or distorted the outer cyst. Excystation occurred when the outer cyst was disrupted and the organism was free (Fig. 8) in the medium. After 4 hr of treatment, the mean ± SE percentage of activated organisms was 61.7 ± 0.9 and 31.7 ± 5.0 for E. revolutum and E. trivolvis, respectively; for breached organisms the mean was 5.0 ± 0.6 and 3.7 ± 0.3 for E. revolutum and E. trivolvis, respectively; and for excysted metacercariae the mean was 30.3 ± 0.9 and 55.7 ± 1.2 for E. revolutum and E. trivolvis, respectively. Student’s t-test (with P < 0.05 being considered significant) showed that the percentage excystation of E. revolutum was significantly less than that of E. trivolvis at 4 hr.

**DISCUSSION**

The cercaria of E. revolutum contains about 4 times the number of paraesophageal glands than that of E. trivolvis (Kanev et al., 1995). Although the role of these glands is not clear during in vivo encystment, their secretions may aid in cyst formation and also in excystation. Following excystation of cysts formed in snails, the excysted metacercariae showed no evidence of paraesophageal gland secretions based on neutral red staining. This was not the case for cysts formed ectopically, where, following excystation, staining of the paraesophageal gland was retained in ducts or openings. The paraesophageal gland ducts or openings of excysted metacercariae of E. trivolvis from either snail origin or from cysts formed ectopically (Fried and Bennett, 1979) do not stain with neutral red.

Abnormalities of cysts formed ectopically were seen in the distorted outer cyst and in the incomplete granular inner cyst. Our knowledge of how echinostome cysts are formed is incomplete, and although some cyst constituents are derived from the cystogenous glands, others may originate from both the penetration and paraesophageal glands. The role of these glands is uncertain and Cleveland and Kearn (1989) suggested that a better name for these glands would be accessory glands. The structure and function of these glands in *E. revolutum* merit further study.

Excystation in *E. trivolvis* and *E. revolutum* is similar in terms of the sequence of events that occur, that is, activation, breaching, and final release of the larva from both cyst walls. The delay in excystation of *E. revolutum* compared to *E. trivolvis* may account in part for microhabitat differences in the gut of the definitive hosts. Echinostoma revolutum is found in the cecum and rectum. In addition to these sites, *E. trivolvis* is also found in the lower ileum. Echinostoma revolutum establishes only in avian hosts, whereas *E. trivolvis* infects both mammalian and avian hosts (see Kanev, 1994; Kanev et al., 1995). Speed of excystation can be a factor in the ability of echinostomes to establish in the gut and rapid gut emptying times may hinder successful excystation and the eventual establishment of newly excysted worms.

Differences in ectopic encystment are apparent in this study compared to that of Fried and Bennett (1979) on *E. trivolvis* (referred to as *E. revolutum* in that study). Fried and Bennett (1979) reported ectopic encystment of *E. trivolvis* on mucus trails of snails, but this did not occur in *E. revolutum*. They also found normal and abnormal cysts in Locke’s 1:1 and in Locke’s 1:1 plus dextrose. We only found abnormal cysts in Locke’s 1:1 plus dextrose. The extent of distortion of the outer cyst and the incomplete formation of the inner cyst of *E. revolutum* in this study contrasted markedly to the structure of ectopic cysts in *E. trivolvis* as reported by Fried and Bennett (1979).

Our findings on encystment of *E. trivolvis* in various snail hosts are not unexpected because echinostome cercariae have broad specificity for their second intermediate snail hosts (Anderson and Fried, 1987; Schmidt and Fried, 1996a). No attempt was made to detect species-specific differences in the snails used as experimental 2nd intermediate hosts for *E. revolutum*.

The finding of *E. revolutum* cysts in newly hatched and juvenile *L. elodes* snails extends observations on encystment in young snails to this echinostome species. Similar findings have been reported for *E. trivolvis* in juvenile *B. glabrata* (Fried et al., 1995), for *E. trivolvis* in juvenile *H. trivolvis* (CO) snails (Schmidt and Fried, 1996a), and for *Echinostoma caproni* in juvenile *B. glabrata* snails (Sullivan, 1985). Mortality associ-
FIGURES 1–8. Light micrographs of *Echinostoma revolutum* encysted and excysted metacercariae and a cercaria. 1. Ectopic cysts formed within 48 hr of cercarial encystment in Locke’s 1:1 plus 1% dextrose. Note presence of tail (t) in 1 cyst but not in the other and distorted outer (o) and incomplete granular inner (i) cyst. 2. Breached larva in ectopic cyst after 30 min in the excystation medium. Note outer (o) and inner (i) cysts. 3. Excysted metacercaria (derived from an ectopically formed cyst) stained with 0.01% neutral red. Note staining associated with ducts of the paraesophageal (pe) and penetration (p) glands. 4. Another excysted metacercaria (derived from an ectopically formed cyst) stained with 0.01% neutral red. Arrows point to the openings of the paraesophageal (pe) glands; note presence of stain in the openings. 5. Cercaria stained in 0.01% neutral red. Note ducts and openings of paraesophageal (pe) glands and penetration (p) glands. 6. Encysted metacercaria from the kidney of an experimentally infected *Helisoma trivolvis* (Colorado strain) snail. Note outer (o) and inner (i) cysts. 7. Breached cyst (of snail origin) of *E. revolutum* after 1 hr in the excystation medium. Note outer (o) and inner (i) cysts. 8. Excysted metacercaria (of snail origin) stained in 0.01% neutral red. Note absence of stain in the ducts and openings of the paraesophageal glands. The scale bars in all figures represent 25 µm.
ated with infection of echinostomes in juvenile snails may be appreciable in the field.

Except for the greater number of paraesophageal glands in *E. revolutum* cercariae compared with *E. trivolvis*, cercariae of these species are difficult to distinguish. Likewise, encysted and excysted metacercariae of these species are difficult to distinguish solely on the basis of morphologic characteristics. Our studies show that there are distinct differences in these species in terms of cercarial ectopic encystment and chemical excystation of their metacercariae.

**LITERATURE CITED**


