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The Inhibitory Effect of Excretory-Secretory Products of *Echinostoma paraensei* Primary Sporocysts on M-line *Biomphalaria glabrata* Hemocytes Motility

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Abstract

Excretory-secretory (E-S) products and worm extracts of cultured *Echinostoma paraensei* primary sporocysts were assayed for their effects on the motility of *Biomphalaria glabrata* hemocytes using chemotaxis chambers equipped with nucleopore membrane filters (pore size 5 μm). The migration rate of hemocytes through the filter in the presence of E-S products, which derived from day 1 cultures or from cultures conditioned from days 2 through 4, was significantly lower than control level. Sporocyst extracts also caused a reduction in the motility. E-S products derived from day 1 cultures were fractionated using ultrafiltration membranes with 10–30-kDa, 30–100-kDa and >100-kDa cut-offs. The migration rate in the presence of 10–30-kDa and 30–100-kDa fractions was significantly lower than control level, while the >100-kDa fraction did not affect the hemocyte migration. These results suggest that echinostome sporocysts produce molecules that interfere with the motility of host snail hemocytes toward parasites.

Key words: *Echinostoma paraensei, Biomphalaria glabrata,* Excretory-secretory products, Motility

Introduction

Echinostoma paraensei has an innate ability to interfere with defense responses of host snail, *Biomphalaria glabrata* (reviewed by Lie, 1982). *In vivo* and *in vitro* studies have indicated that hemocytes from snails infected with *E. paraensei* have reduced ability to encapsulate and destroy larvae of *Schistosoma mansoni* (Lie *et al.*, 1977; Loker *et al.*, 1986). *E. paraensei*-infected snails contained larger numbers of hemocytes with less ability to adhere to foreign surfaces (Noda and Loker, 1989a), and hemocytes taken from infected snails have low phagocytic activity (Noda and Loker, 1989b). Excretory-secretory (E-S) products released from echinostomes may have the ability to alter a variety of hemocyte functions. Phagocytosis assays indicated that E-S products of *E. paraensei* strongly inhibited uptake of sheep red blood cells by *B. glabrata* hemocytes (Loker *et al.*, 1992). Also, hemocytes pretreated with the E-S products assumed a spherical shape and failed to spread normally (Loker *et al.*, 1992). E-S products of *S. mansoni* inhibited the motility of *B. glabrata* hemocytes (Lodes and Yoshino, 1990). Migration of host snail hemocytes toward parasites may play an important role in the molluscan defense system. In the present study, the effect of *E. paraensei* E-S products on the motility of host snail hemocytes was investigated using blind well chambers.

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Materials and Methods

Animals and parasite

E. paraensei was maintained in hamsters and M-line *B. glabrata* as described by Loker and Hertel (1987). Eggs of *E. paraensei* were harvested from the uteri of adult worms and maintained in artificial spring water (Ulmer, 1970). All snails employed in the experiments were 13–15 mm in shell diameter.

Culture of sporocysts and collection of E-S products

The culture medium consisted of a 1:1 mixture of

half-strength medium 199 and Bge medium (Bayne *et al.*, 1977) that was buffered at pH 7.2 with 25 mM HEPES. Eggs of *E. paraensei* were maintained in the dark at 26°C for 16 days prior to hatching. Eggs were then suspended in 1 ml culture medium, and they were exposed to light. Hatched larvae were transferred to sterile 1.5-ml plastic tubes and cultured at concentrations ranging from 5,000 to 15,000 sporocysts/ml at 26°C. The conditioned medium containing sporocyst E-S products was collected at day 1. Sporocysts were then suspended in the fresh medium, and the conditioned medium was collected again at day 4.

Conditioned medium was centrifuged at 2,000 g for 5 min to removed particulate materials. The supernatant from 30,000 sporocysts was concentrated to 500 μl using an ultrafiltration membrane (10-kDa cutoff filter, Nihon Millipore Limited, Tokyo). This solution was used as E-S products of sporocysts. The remaining sporocysts were washed with the culture medium 2 times and resuspended in the fresh culture medium. The parasites were homogenized with a glass homogenizer and centrifuged at 2,000 g for 5 min. The supernatant was concentrated to 500 μl using an ultrafiltration membrane and used as extracts of sporocysts.

E-S products collected on day 1 were separated into 3 molecular weight fractions using ultrafiltration membranes (10-kDa, 30-kDa, and 100-kDa cutoff filters, Nihon Millipore Limited): 10–30-kDa, 30–100-kDa, and >100-kDa fractions. Each fraction was concentrated at 500 μl using an ultrafiltration membranes.

Hemocyte migration assay

Motility of hemocytes was measured using blind well chambers (BW25: Neuro Probe, Inc., Cabin John, Maryland) and PVP-free nucleopore membrane filters (5 μm pore size: Neuro Probe, Inc.) (Fig. 1). More than 80 μl hemolymph was obtained from individual snails by cardiac puncture (Loker and Hertel, 1987), and placed on parafilm. A 20 μl aliquot of each hemolymph sample was transferred to 500-μl tubes and centrifuged at 2,000 g for 5 min. After centrifugation, 18 μl of the resultant plasma was mixed 1:1 with the appropriate test medium or culture medium and pipetted into the well of the lower chamber, and a filter was placed. The upper chamber was then applied. The remaining 60 μl portion of hemolymph containing hemocytes was mixed 1:1 with culture medium and pipetted into the well of the upper chamber. The chamber was sealed with parafilm and incubated at 26°C for 2 hrs. After incubation, the filter was removed and placed on a glycerol-coated glassslide with the lower surface facing upwards and was air-dried for 60 min. Each

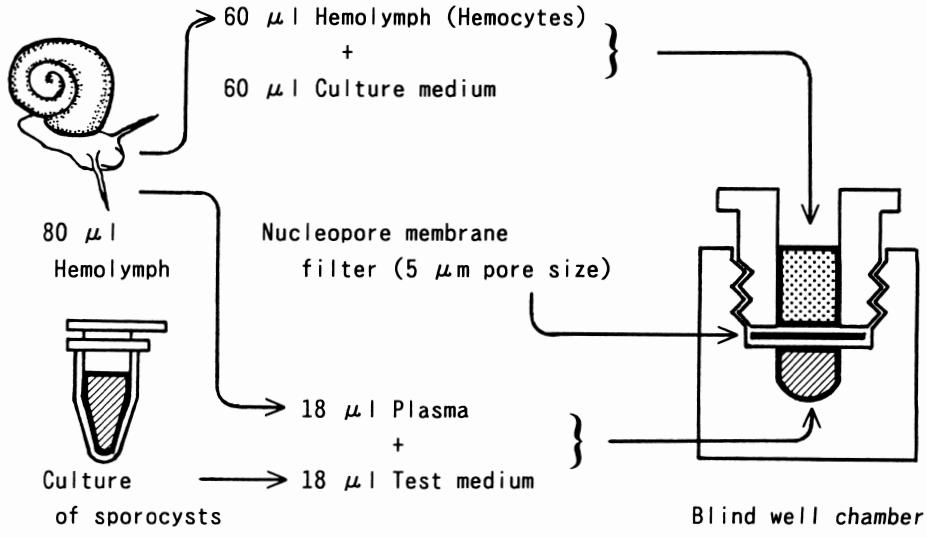

Fig. 1. Hemocyte migration assay protocol.

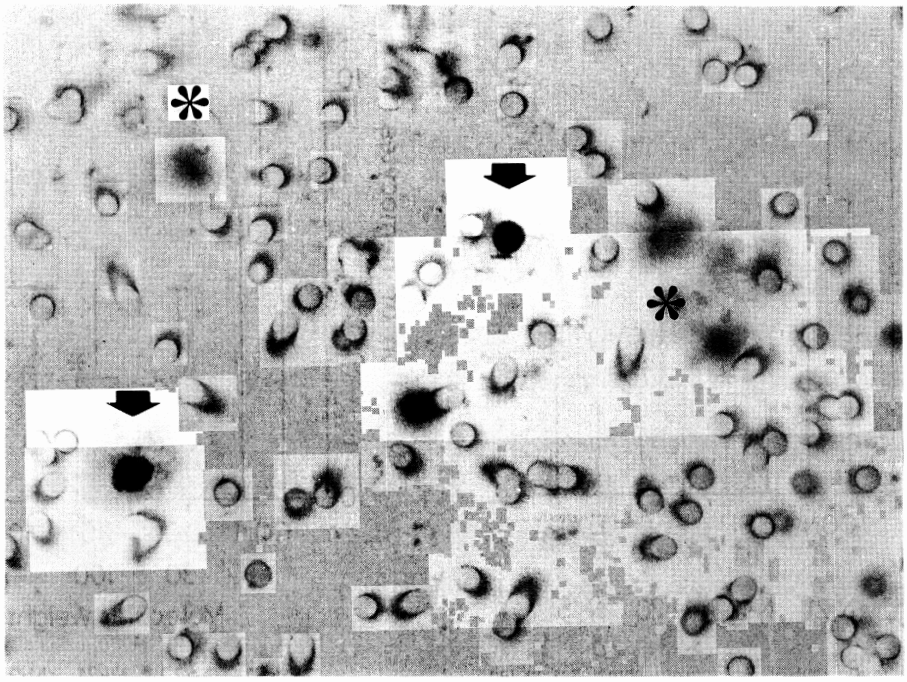

Fig. 2. *B. glabrata* hemocytes on a 5 μm nucleopore membrane filter. Arrows show hemocytes which have migrated through the filter. Unclear dark spots marked by asterisks are hemocytes which stayed on the upper surface of the filter.

filter was fixed with methanol and stained with Giemsa's stain solution. Hemocytes attached on both surfaces of the filters were counted for 200 randomly selected oil immersion fields, and the migration rate of hemocytes was calculated; the migrated cell number was divided by the total cell number (Fig. 2). In this system, the migration rate indicates the level of motility of hemocytes.

Statistical analysis

Student's *t*-test was used for comparison of means. Results were accepted as significant at $P<0.05$.

Results

The migration rate of *B. glabrata* hemocytes into the lower well in the presence of culture medium (control) was 7.0±3.9%. Microscopical examination of the lower wells of chamber revealed that no hemocytes migrated through and detached from the lower surface of the filter. The effect of sporocyst E-S products and extracts on the migration of hemocytes is shown in Fig. 3. The motility of hemocytes in the presence of sporocyst E-S products was significantly lower than the control, both for E-S products from day 1 cultures and from cultures conditioned from days 2 through 4. Sporocysts extract also significantly reduced the migration rate of hemocytes below control levels.

The effect of fractionated E-S products (10–30-kDa, 30–100-kDa and >100-kDa fractions) derived from day 1 cultures on the migration rate of hemocytes is shown in Fig. 4. The 10–30-kDa and 30–100-kDa fractions significantly reduced hemocyte motility.

Discussion

The larval stage of some trematodes interferes with hemocyte functions. This interference seems to be an important survival mechanism of trematode larvae. Lie (1982) suggested that susceptibility of snails to some trematode species may be due to interference of host defense reaction by secretory

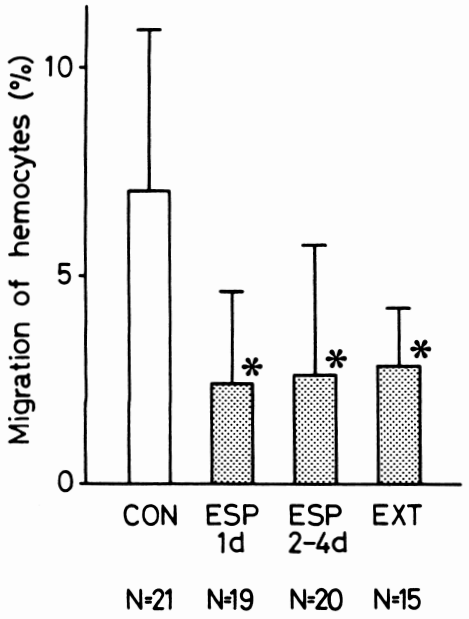

Fig. 3. Migration rate of *B. glabrata* hemocytes in the presence of control medium (control), E-S products and extracts of *E. paraensei* sporocysts. Each bar shows the mean ± SD. Abbreviations: CON, control medium; ESP 1d, E-S products derived from day 1 cultures; ESP 2–4d, E-S products derived from cultures conditioned from days 2 through 4; EXT, extracts of sporocysts. N indicates the number of assay replicates. Values marked by an asterisk differ significantly from control (Student's *t*-test, $P<0.05$).

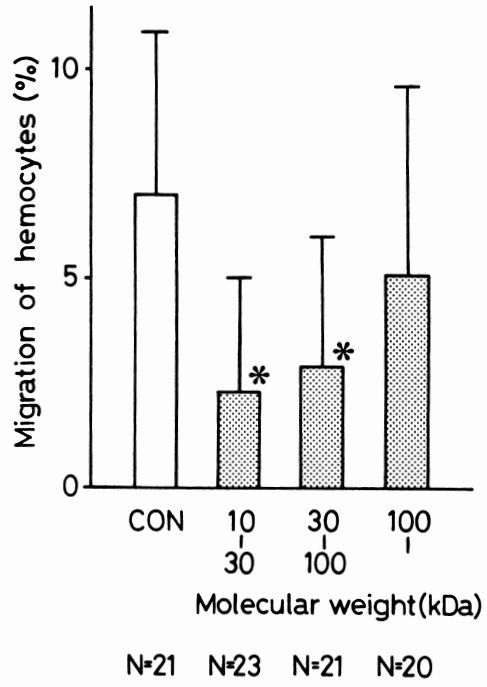

Fig. 4. Migration rate of *B. glabrata* hemocytes in the presence of fractionated E-S products (10–30-kDa, 30–100-kDa, and >100-kDa fractions) of *E. paraensei* sporocysts. Culture medium was used for control (CON). Each bar shows the mean ± SD. N indicates the number of assay replicates. Values marked by an asterisk differ significantly from controls (Student's *t*-test, $P<0.05$).

products released from larvae. Phagocytosis and encapsulation are considered the most common forms of cellular reactions of molluscs in response to invading parasites and bacteria, and these processes involve the migration of hemocytes toward the foreign particles. Schmid (1975) demonstrated that hemocytes of gastropod *Viviparus malleatus* were attracted chemotactically to heat-killed *Staphylococcus aureus* and to N-acetyl-D-glucosamine. Hemocytes of American oyster, *Crassostrea virginica,* were attracted to live *Escherichia coli, Bacillus megaterium,* and *Micrococcus varians* (Cheng and Howland, 1979). Hemocytes of two marine neritid gastropods, *Nerita albicilla* and *Heminerita japonica*, and of the brackish-water clam, *Corbicula japonica*, were attracted chemotactically to live *Vibrio parahaemolyticus* and *E. coli* (Kumazawa *et al.*, 1992; Kumazawa and Morimoto, 1992). These results indicate that molluscan hemocytes possess the ability to respond to chemotactic stimulation. In the present study, *E. paraensei* sporocyst E-S products inhibited the motility of *B. glabrata* hemocytes. This suppressive activity was found in conditioned medium derived from day 1 cultures and from medium conditioned from days 2 through 4. These results suggest that echinostome sporocysts produce molecules that interfere with the motility of host snail hemocytes toward parasites.

Loker *et al.* (1992) characterized E-S products of *E. paraensei* sporocysts. Miracidia of *E. paraensei* were cultured in medium containing ^{14}C-labeled amino acids. E-S products collected during day 1 of culture contained several polypeptides, none of which were labeled, suggesting that these E-S products are largely performed. E-S products released by

sporocysts in day 2 and older cultures were generally similar and contained several polypeptides, many of which were labeled, indicating active synthesis of E-S products. These observations indicate that *E. paraensei* sporocysts actively synthesize and release molecules which interfere with the motility of hemocytes in the host snail. Lodes and Yoshino (1990) reported that E-S products of *S. mansoni* derived from initial 24 hr culture, but not those products released by 8-day sporocysts, significantly inhibited the motility of hemocytes from a strain of *B. glabrata* susceptible to *S. mansoni*. These facts may be related to the strong ability of *E. paraensei* to interfere with the host snail's defense reaction (Lie *et al.*, 1977; Lie, 1982).

In this study, both the 10–30-kDa and the 30–100-kDa fractions of E-S products significantly inhibited the motility of hemocytes. Similar inhibitory activity was found in both high (>30-kDa) and low (<10-kDa) fractions of *S. mansoni* E-S products but not in an intermediate fraction (10–30-kDa) (Lodes and Yoshino, 1990). These results may indicate that multiple components in E-S products of trematode larvae have the ability to inhibit the motility of hemocytes. Lodes and Yoshino (1990) showed that *S. mansoni* sporocyst E-S products differentially affect the motility of hemocytes from susceptible strain (M-line) and a resistant strain (10-R2-OK) of *B. glabrata*. They suggested that differential susceptibility of *B. glabrata* to schistosome larvae could, in part, be due to interference of host snail hemocyte motility by parasite E-S products. Further studies are required to characterize E-S products from echinostomes and to determine how E-S products affect the motility of host snail hemocytes.

Acknowledgments

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References

1) Bayne, C. J., Chao, J. and Salvatore, P. (1977): Low-temperature preservation of the *Biomphalaria glabrata* cell lines. J. Invertebr. Pathol., 29, 332–337.
2) Cheng, T. C. and Howland, K. H. (1979): Chemotactic attraction between hemocytes of the oyster, *Crassostrea virginica*, and bacteria. J. Invertebr. Pathol., 33, 204–210.
3) Kumazawa, N. H., Iwao, K. and Morimoto, N. (1992): Chemotactic activity of hemocytes derived from two marine neritid gastropod molluscs, *Nerita albicilla* and *Heminerita japonica*, to *Vibrio parahaemolyticus* and *Escherichia coli* strains. Jpn. J. Vet. Sci., 54, 243–247.
4) Kumazawa, N. H. and Morimoto, N. (1992): Chemotactic activity of hemocytes derived from a brackish-water clam, *Corbicula japonica*, to *Vibrio parahaemolyticus* and *Escherichia coli* strains. Jpn. J. Vet. Sci., 54, 851–855.
5) Lie, K. J. (1982): Survival of *Schistosoma mansoni* and other trematode larvae in the snail *Biomphalaria glabrata*. A discussion of the interference hypothesis. Trop. Geog. Med., 34, 111–122.
6) Lie, K. J., Heyneman, D. and Richards, C. S. (1977): Studies on the resistance in snails: Interference by nonirradiated echinostome larvae with natural resistance to *Schistosoma mansoni* in *Biomphalaria glabrata*. J. Invertebr. Pathol., 29, 118–125.
7) Lodes, M. J. and Yoshino, T. P. (1990): The effect of schistosome excretory-secretory products on *Biomphalaria glabrata* hemocyte motility. J. Invertebr. Pathol., 56, 75–85.
8) Loker, E. S., Bayne, C. J. and Yui, M. A. (1986): *Echinostoma paraensei*: Hemocytes of *Biomphalaria glabrata* as targets of echinostoma mediated interference with host snail resistance to *Schistosoma mansoni*. Exp. Parasitol., 62, 149–154.
9) Loker, E. S., Cimino, D. F. and Hertel, L. A. (1992): Excretory-secretory products of *Echinostoma paraensei* sporocysts mediate interference with *Biomphalaria glabrata* hemocyte function. J. Parasitol., 78, 104–115.
10) Loker, E. S. and Hertel, L. A. (1987): Alterations in *Biomphalaria glabrata* plasma induced by infection with the digenetic trematode *Echinostoma paraensei*. J. Parasitol., 73, 503–513.
11) Noda, S. and Loker, E. S. (1989a): Effects of infection with *Echinostoma paraensei* on the circulating haemocyte population of the host snail *Biomphalaria glabrata*. Parasitol., 98, 35–41.
12) Noda, S. and Loker, E. S. (1989b): Phagocytic activity of hemocytes of M-line *Biomphalaria glabrata* snails: Effect of exposure to the trematode *Echinostoma paraensei*. J. Parasitol., 75, 261–269.
13) Schmid, L. S. (1975): Chemotaxis of hemocytes from the snail *Viviparus malleatus*. J. Invertebr. Pathol., 25, 125–131.
14) Ulmer, M. J. (1970): Notes on rearing snails in the laboratory. In experiments and techniques in parasitology, MacInnis, A. J. and Voge, M., ed., W. H. Freeman and Co., Sanfrancisco, 143–144.