Ultrastructural Study of the in Vitro Interaction between *Biomphalaria glabrata* Hemocytes and *Schistosoma mansoni* Miracidia

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Biomphalaria glabrata *and* Schistosoma mansoni *relationship was studied by light microscopy (LM) and freeze-fracture replica technique (FFR). We observed very thin cytoplasmic extensions of hemocytes in the LM, which then surround immobilize the miracidia. FFR images showed that the contact site between hemocytes cytoplasmic extensions and the external tegumentary coat involved only superficial layers of miracidia. Numerous vacuoles and filopodia were observed in the hemocyte cytoplasm, the latter binding with those from neighboring cells. In spite of the close interfilopodia contact, no cellular junctions were seen at these sites nor between filopodia-miracidia contact areas. The observed migration of hemocytes and their disposition in layers surrounding the miracidia in vitro correspond to previous studies.*

Key words: *Biomphalaria glabrata* - *Schistosoma mansoni* - freeze-fracture replica

The blood fluke, *Schistosoma mansoni* causes schistosomiasis in man and rodents. This disorder is characterized by the presence of *S. mansoni* eggs in the host liver and intestinal tissues. *Biomphalaria glabrata*, a freshwater planiform mollusc, is the main intermediate host species for *S. mansoni* (Rey 1992). In the invertebrate host, *S. mansoni* grows polyembryonically (Van der Knaap & Loker 1990, Barrios et al. 2001). The cycle starts in water when *S. mansoni* miracidia penetrate soft regions of the mollusc, lose their ciliated epithelium and become sack-like structures, the sporocysts, constituted internally by germinative or reproductive cells (Coelho 1995, Sapp & Loker 2000). Several studies have associated the main defense line in *B. glabrata* against *S. mansoni* infection with humoral and cellular factors (Ottaviani & Tarugi 1986), especially hemocytes present in the mollusc hemolymph (Sapp & Loker 2000). *B. glabrata* hemocytes are capable of binding and destroying the parasite larvae by (1) phagocytosis of the tegument, (2) liberation of cytotoxic components or (3) both mechanisms simultaneously (Adema & Loker 1997, Hampton et al. 1998). These defense mechanisms have been described for resistent molluscs whereas in susceptible molluscs, the hemocytes bind to the parasites in a transitory and inefficient way allowing the successful evolution of the parasite (Vasquez & Sullivan 2001).

Financial support: Fonacit UCGEI-2000001901, CDCH-UCV 03-10-4169-2000, and CDCH-UC 99014
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Received 7 April 2003
Accepted 8 October 2003

Morphological and ultrastructural methods previously employed for the characterization of vertebrate immune systems (macrophages, lymphocytes, and polymorphonuclears) under normal conditions and parasitic infections were used for identifying mollusc hemocytes (Sminia & Barendsen 1980, Ottaviani & Franchini 1988, Abdul-Salam & Michelson 1980, Barracco et al. 1993). It has been lately suggested that some bacteria toxins are able to induce the re-arranging of Rho superfamily proteins and cytoskeleton alterations (stress fibers) in epithelial cells (Brest et al. 2003). These findings indicate a new perspective for studying the mechanisms mediating the *B. glabrata* hemocyte and *S. mansoni* larval interaction.

The aim of this investigation was to study ultrastructural aspects of the interaction between *B. glabrata* hemocyte and *S. mansoni* occurring at the cellular membrane level. For this purpose, a useful methodology for the ultrastructural characterization of membrane systems, the freeze-fracture replica technique (FFR) was used.

MATERIALS AND METHODS

Miracidia - *S. mansoni* eggs were purified from the liver and gut sections of hamsters (*Mesocricetus auratus*) 7 weeks after infection. Briefly, dissected organs were enzymatically digested (0.25% trypsin and 0.05% collagenase in 0.15 M PBS, pH 7.2) then eggs were purified by shifting through metallic meshes, decreasing in size from 420 to 44 µm. Eggs were collected and hatched under sterile conditions, and the resulting miracidia were sedimented at 4ºC as described by César and Alarcón de Noya (1987). All the animals used in this study were sacrificed in accordance to Venezuelan laws for animal care.

Cytoadherence - Hemocytes were obtained from *B. glabrata* between 7 and 10 mm diameter. Molluscs were externally rinsed with 25% isopropylic alcohol and then

washed three times in a solution containing 100 µg streptomycin and 100 IU/ml penicillin for 20 min each time. After external drying, molluscs were placed in warm water and hemolymph was collected by cephalopodal puncture. In order to induce the binding of the hemocytes on glass, 125 µl hemolymph were placed on glass microslides and incubated in an humid chamber at 27°C during 2 h.

In vitro interaction assay - Was done by adding 100 miracidia/100 µl PBS to each microslide. Samples were incubated for 90 min at 27°C, then evaluated, and images were taken using a Nikon E400 light microscope and FFR in an electron transmission microscope.

In vivo assay - Was carried out on snails between 4-5 weeks after infection; they were dissected and the cephalopodal portion was removed. The replicas were obtained from samples fixed in 2% glutaraldehyde solution in PBS during 30 min. Samples were gently scraped with a wooden stick from the glass coversheets, cryoprotected in 25% glycerol in PBS, mounted on replica hats and finally, frozen by immersion in undercooled liquid nitrogen. Frozen samples were kept in liquid nitrogen until use. Frozen samples were cryofractured in a JEOL 9010C freeze-fracture device ($1x10^{-6}$Pa, -120°C), shadowed with PT/C and coated with C. Replicas were cleaned in 50% chlorox overnight at room temperature, rinsed in distilled water, and collected on 400 mesh copper grids. They were observed in a JEOL transmission electron microscope (JEM Mod. 1220) at 120 kV.

RESULTS

The in vitro observation of the early events of miracidia-hemocyte interaction showed three relevant aspects: (1) the migration of the hemocytes towards the proximity of the miracidia; (2) the emission of very thin cytoplasmic extensions of hemocytes (Fig. 1), and (3) a confluence of hemocytes on the miracidia which is finally and totally immobilized (Fig. 2). The light microscopic images showed hemocytes close to miracidia, very thin cytoplasmic extensions which completely surround miracidia before finally immobilizing (Fig. 2). By FFR, hemocytes appear to have a cytoplasm with irregular and sinuous contours containing abundant mitochondria and autophagic vacuoles. We also observed numerous intra-cytoplasmic components as well as some binding areas between hemocytes and the hemocyte cytoplasm revealed abundant organelles and cytoplasmic prolongations; some focal contact points were observed between hemocyte cytoplasmic extensions and the external tegumentary layer of the miracidia (Fig. 3).

The in vivo interaction showed a complex structure similar to a cytoplasmic network surrounding the miracidia (Fig. 4). Internal, middle and external layers of the tegumentary miracidia remained intact (Fig. 5).

DISCUSSION

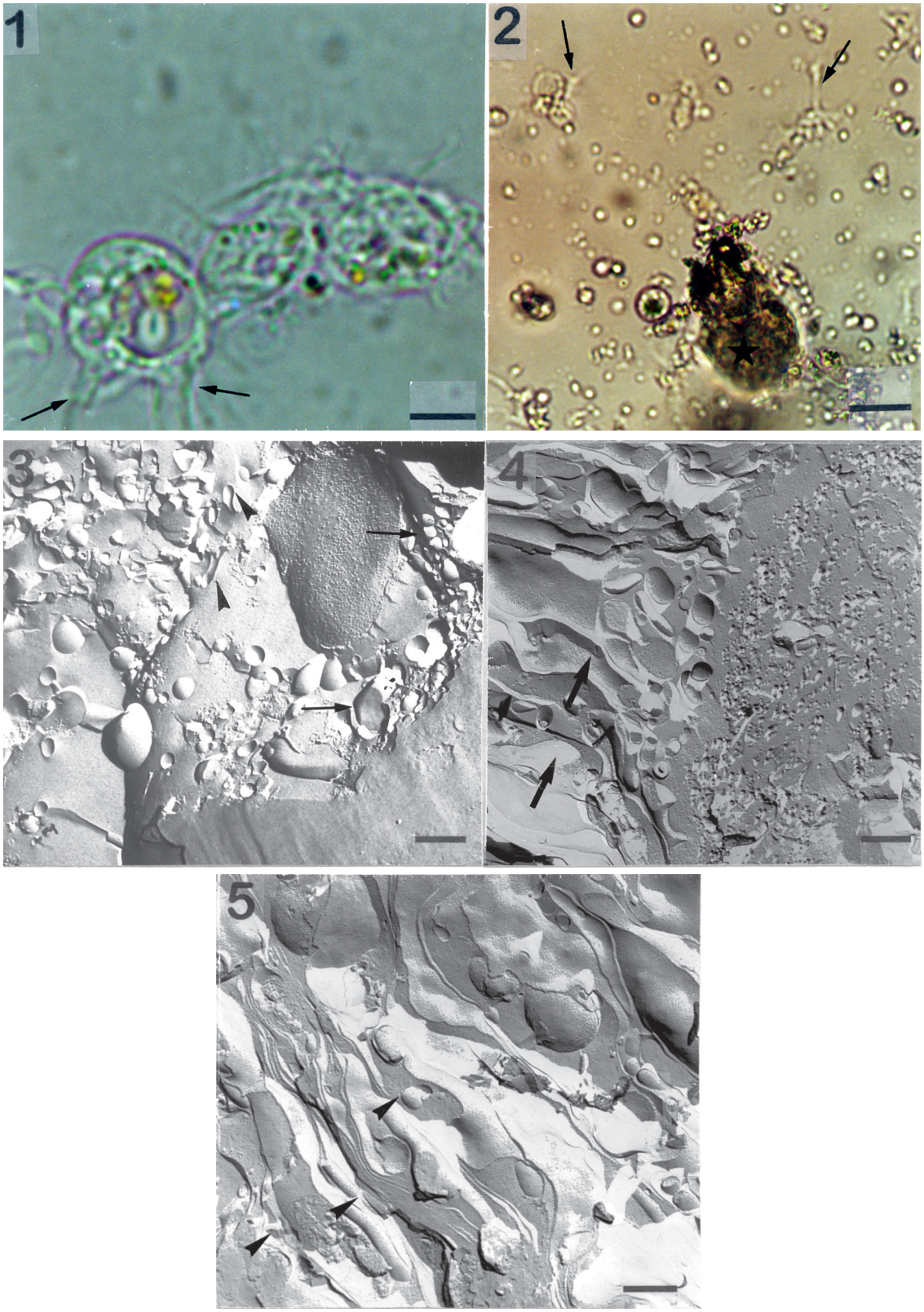
Hemocytic activity in *B. glabrata* is stimulated by several factors, such as secretory/excretory products from *S. mansoni* miracidia. The invertebrate host defense response may include migration, super-oxide anions production associated with an encapsulation process or cell-mediated cytotoxicity reactions (Dikkeboom et al. 1988, Adema et al. 1994, Loverde 1998). All of these responses can occur indifferently in both resistant and susceptible molluscs, especially at the beginning of the interaction. It has been demonstrated that the fundamental difference between resistance or susceptibility is the ability of the parasite to inactivate the hemocyte migration towards the miracidia (Van der Knaap & Loker 1990, Hahn et al. 2001).

Light microscopy indicated that hemocytes migrate towards miracidia, after which very thin cytoplasmic extensions develop around the miracidia and finally the *S. mansoni* larva is completely surrounded. The present observation corresponds to early stages of encapsulation classically observed in infected mollusc tissues (Sapp & Loker 2000). The observed multilayer hemocyte arrangement around the miracidia corresponds with the encapsulation response in mollusc tissues (Loker & Bayne 1982). Morphological characteristics of migrating hemocytes seem to indicate that these cells are a subpopulation of type II hemocyte granulocytes (Delgado et al. 2001). The presence of numerous vesicles and RER in those hemocytes surrounding the miracidia could be associated with increased protein synthesis and production of toxic radicals as has been previously described for studies in vitro (Sapp & Loker 2000). In contrast with ultrastructural changes observed in hemocyte activation processes, the miracidia lost their external ciliated layer, but no changes were evident as regards the internal architecture of larva. This fact can be explained as an insufficient interaction time between hemocyte and miracidia (Coelho 1995). It has been demonstrated that the effectiveness of the parasitic infection process is based on two factors. First, the ability of the parasite to synthesize antigenic determinants similar to those of the mollusc, a process known as molecular mimesis, and secondly, the absorption and incorporation of mollusc agglutinins and hemolymph soluble components to the tegument surface, a process known as antigenic masking (Coelho 1995).

Our results support the hypothesis that hemocytes migrate, independent on their compatibility with larval miracidia and, that migrations towards more compatible larval are less efficient and the interaction hemocyte-parasite less effective (Sapp & Loker 2000, Montesdeoca et al. 2002). We also show that the multilayer cell arrangement around miracidia is consistent with the observed encapsulation response in mollusc tissues (Loker et al. 1982). Electron microscopy has proved to be a successful tool for studying the early stages of hemocyte-miracidia interaction. A more complete study, employing *B. glabrata* hemocytes incompatible with *S. mansoni* simultaneously interacting with *S. mansoni* miracidia in a toxic radical medium should be undertaken.

ACKNOWLEDGMENTS

To Olga Ojeda, Jennifer Ayala, Lic Viana Pinto, Dr Frances Osborn, Prof. Carmen Rodríguez for technical assistance on english suggestions and to the Grupo de Polímeros at the Universidad Simón Bolívar, Venezuela for the use of the transmission electronic microscopy.



Figs 1, 2: light microscope images of the hemocyte-miracidia interaction. It shows the very thin cytoplasmic prolongations (Fig. 1, arrows) and the confluence of hemocytes (Fig. 2, arrows) around miracidia (Fig. 2, star). Figs 3, 5: freeze-fracture replica images. Irregular and sinuous contours were evident in the hemocyte cytoplasm (Fig. 3, arrowheads), abundant mitochondria, autophagic vacuoles, and numerous intra-cytoplasmic componens (Fig. 3, small arrows). Observe the network-like hemocyte cytoplasm surrounding the miracidia (Fig. 4, large arrows). Layers of miracidia tegument can also be seen (Fig. 5, arrowheads). Bars: Fig. 1: 33 µm. Figs 2: 3 µm. Figs 3-5: 500 nm

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