Extra-cellular Matrix Changes in *Schistosoma mansoni*-infected *Biomphalaria glabrata*

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Reactivity of snails against parasites exhibits a primitive focal reaction, with encapsulation, phagocytosis and destruction of parasite larvae by macrophage-like cells – the hemocytes. This reaction mimics granulomatous inflammation seen in higher animals. However, different from the latter, little is known about the participation of extra-cellular matrix in such snail defense reactions. Normal and Schistosoma mansoni*-infected* Biomphalaria glabrata *of different strains were submitted to cytological, histological, ultrastructural and biochemical methods in order to investigate the behavior of extra-cellular tissues at the site of anti-parasite reactions. In spite of the presence of two cell-types in peripheral hemolymph, only one cell-type was present at the sites of tissue reactions. Although pre-existent collagen and elastic fibers and microfibrils sometimes appeared slightly compressed around focal reactions, no evidences of duplication, synthesis or deposition of connective-tissue extra-cellular components were observed within or around the zones of reactive cell accumulations. Thus, tissue reactions against* S. mansoni *in the snail* B. glabrata *appeared exclusively dependent on one specific population of hemocytes.*

Key words: *Biomphalaria glabrata* - *Schistosoma mansoni* - hemocytes - encapsulating complexes - extra-cellular matrix

Many parasites, including several species of medical and veterinary significance, have life cycles that require development, reproduction or both in invertebrate hosts. These hosts also vigorously defend their self-integrity (Loker 1994). In common with other invertebrate groups, mollusks lack an adaptative immune system, with specific antibodies and memory cells of vertebrates. Their internal defense system comprises humoral and cellular elements cooperating in the recognition and destruction of invading organisms (Ouwe et al. 1994). Several authors have recognized the existence of subpopulations of hemocytes, depending on their age (Dikkeboom et al. 1984), their enzyme content (Granath & Yoshino 1983), or their surface determinants (Dikkeboom et al. 1985, Yoshino & Granath 1985). Resistant *Biomphalaria glabrata* specimens typically encapsulate larval stages of nonadapted invading parasites, such as the sporocysts of schistosomes, in a structure that morphologically resembles a primitive granuloma, the encapsulating complexes (Pan 1965, Lie & Heyneman 1980, Jeong & Heyneman 1984). Since the participation of connective-tissue cells and matrix is so prominent in the granulomas of higher animals, one wonders whether a primitive form of participation of these elements would be expressed in mollusks. As noted by the same authors just mentioned, extracellular fibrils were observed in association with the molluskan granuloma-like structures. Lie and Heyneman (1980) did observe fibrils with a periodicity of 80 nm in the outermost layer of capsules containing daughter sporocysts in snails undergoing self-cure. Sminia et al. (1974) reported deposition of collagen-like fibrils at the periphery of encapsulating reactions to *Lymnaea stagnalis*, by showing that the flattened cells of the outer capsule layers were involved in the synthesis of these fibrils. Also, Rifkin et al. (1969), Yoshino (1976), and Krupa et al. (1977) noted the presence of extracellular fibrils in association with molluscan cellular responses, but Harris (1975) found no extracellular elements contributing to the formation of capsules.

The present paper extends these observations by documenting aspects of hemocytes and extracellular matrix and connective-tissue cells in normal and in *Schistosoma mansoni*-infected *B. glabrata*, representative of. strains with variable degrees of resistance/susceptibility to the parasite.

MATERIALS AND METHODS

Snails and exposure to S. mansoni miracidia - Susceptible (Feira de Santana-FS, BA, Brazil) and resistant *B. glabrata* (Barreiro de Cima, Belo Horizonte-BH, MG, Brazil) to *S. mansoni* were maintained in aerated water at 28°C and fed on lettuce ad libitum. The snails measured from 8 to 16 mm in shell diameter. A total of 30 snails (FS) were submitted to infection with 20 miracidia, during 1 h with a Feira de Santana strain of *S. mansoni* (Andrade & Sadigurky 1985). Ten specimens from Minas Gerais were exposed individually to 50 miracidia with a BH strain of *S. mansoni*. Following exposure to miracidia, all snails were maintained in a dark room for 35 days, the water being changed twice weekly.

Cercarial shedding - Emission of cercariae occurred at the first month following exposure. Cercarial release was monitored by placing the snails, individually, in 100 ml beakers containing 10 ml of dechlorinated tap water. Each specimen was exposed to bright light during 1 h, for cercarial shedding and counting. The exposed snails were classified according to whether they were emitting cer-

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cariae: a group eliminating from zero to 10 cercariae was considered as poorly susceptible; from 50 to 150: as susceptible, and from 151 to 500, extremely susceptible. Non-exposed snails were used as controls.

Histology - Snails were examined at different days pos-infection (24 h up to 100 days). They were submitted to anesthesia with menthol crystals for 4 h before being removed from the shells. The entire snail was placed in Bouin's fluid during 5 h for fixation and then transferred to 70% alcohol. Further procedures included dehydration in 100% alcohol, clearing in xylol and embedding in paraffin. Sections were stained with hematoxylin and eosin, sirius-red method for collagen, orcein and Weigert's resorcin-fucsin for elastic fibers. Sirius-red stained slides were microscopically examined with and without polarizing filters (Junqueira et al. 1979).

Electron microscopy - After removal from the shell, the snail was cut in small pieces and immediately fixed in iced 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h and post fixed in 2% osmium tetroxide in 0.2 M phosphate buffer. Specimens were dehydrated in acetone and embedded for 12 h in Poly-bed 812 resin. Ultra thin sections from selected blocks were made with a diamond knife in a Reichert-Supernova ultra-microtome. Sections were mounted on copper grids, contrasted with uranyl acetate and lead citrate, and examined with a Zeiss Electron Microscope at 50 Kv.

Collagen measurements - The method of Bergman and Loxley (1963) was used to determine collagen concentration by the measurement of hydroxyproline content. Briefly, after fixation in neutral 10% formalin the snails were hydrolyzed for 9 to 18 h in hydrochloric acid at 110°C, neutralized in NaOH (10N) and HCl (6N). Samples of 100 µl were treated in mixture of chloramine T and Erlichs' reagent and read in a Hitachi 200 spectrophotometer (558 nm). As positive controls, fragments of a fibrotic mouse liver (*S. mansoni*-infected) were used.

Cytology - The examination of circulating hemocytes was made from a fresh hemolymph sample. To obtain hemocytes, four snails were selected for each group. Snails were first swabbed with 70% ethanol to remove debris from the external surface and then the shell was perforated with a needle (13 x 4 mm). The iced-samples were collected and placed into a 500 µl siliconized microcentrifuge tube for 5 min to allow shell debris or mucus to sediment. After sedimentation, slides with 10 ml of hemolymph were incubated at 22-28°C for 30 min, fixed in methanol for 5 min and stained with Giemsa or Neutral red.

RESULTS

Sections from infected snails invariably presented focal collections of sporocysts and cercariae in several developmental stages, as well as in several degrees of disintegration, usually surrounded by focal and diffuse accumulations of hemocytes. The number of parasite collections and the extent of hemocyte proliferation appeared to be inversely proportional to the degree of snail susceptibility or resistance of the strains, as can be appreciate in Fig. 1A, B. Apart from that, the type of tissue reaction was the same for all parasitized snails. Location of the lesions did not differ, either. The sites mostly parasitized were the digestive glands, ovo-testis and the renal regions. The capsular reactions exhibited distinct slightly flattened hemocytes, many of which contained phagocytosed material from sporocysts and cercariae. Focal hemocyte concentrations around groups of proliferating as well as disintegrating parasites assumed the arrangement of a granuloma, appearing at the routinely stained slides as a mixture of cells and fibers (Fig. 2A, B, C). Although special staining for collagen and elastic tissue failed to reveal specific fibers in these granuloma-like lesions, collagen and rare elastic fibers seemed to form an outer, thin and incomplete capsule around the focal en-

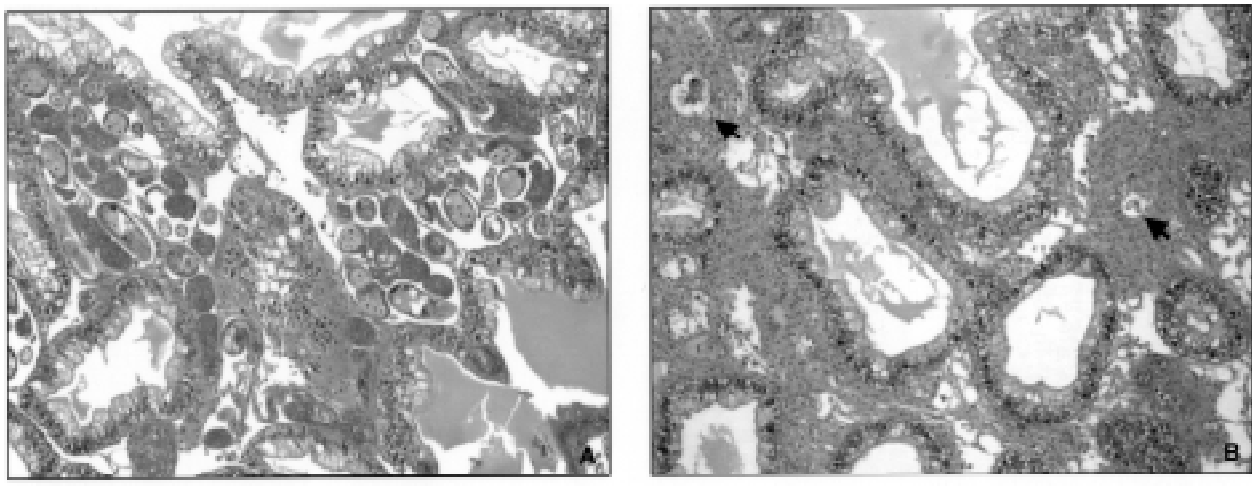

Fig. 1A: section from the digestive glands of a highly susceptible *Biomphalaria glabrata* showing numerous developing forms of *Schistosoma mansoni* and the almost total absence of cellular reaction; B: extensive and diffuse cellular reaction within the connective tissue, and concentrically arranged around a few disintegrating portions of the parasite (arrow) in the digestive glands of a *S. mansoni* resistant snail. Hematoxylin & Eosin, 100X

capsulated lesions. In effect, closer examination detected some rather slightly compressed pre-formed fibers (Fig. 2D). Sometimes hemocyte proliferation resulted in diffuse collections of such cells, devoid of any fibers.

At the ultrastructural level, collections of hemocytes were represented by cells with numerous interdigitating filopodia, forming a complex network of cytoplasmic prolongations, in the presence of an amorphous, poorly dense matrix, but in the absence of cellular or fibrous elements (Fig. 3A, B). Some of these cells showed a large nucleus with the chromatin distributed along the nuclear periphery (Fig. 3B, C). Mitochondria, Golgi apparatus, electron-dense bodies (lysosomes) and myelinoid bodies were regularly found. At the proximity of the hemocyte collections some parallel disposed fibers and fibrils were noted (Fig. 2D). Some of these fibers revealed periodicity, suggesting a collagenous nature.

No significant differences were noted in normal and *S. mansoni*-infected snails submitted to biochemical determination of hydroxyproline.

Two kinds of hemocytes were found in fresh hemolymph samples: small spherical hemocytes with a large nucleus surrounded by a thin cytoplasm layer (they adhered to glass surface, but did not spread appreciably), and larger spreading hemocytes with extending numerous thin filopodia. However, in the capsules observed in the kidney and digestive glands, only spreading hemocytes could be positively identified. The number of spreading cells present in hemolymph of snails at 24 h-35 days after infection did not differ quantitatively after a simple counting on stained slides, but it did diminish at 49-100 days.

The normal non-infected snails showed essentially normal histology. In areas with abundant connective tis-

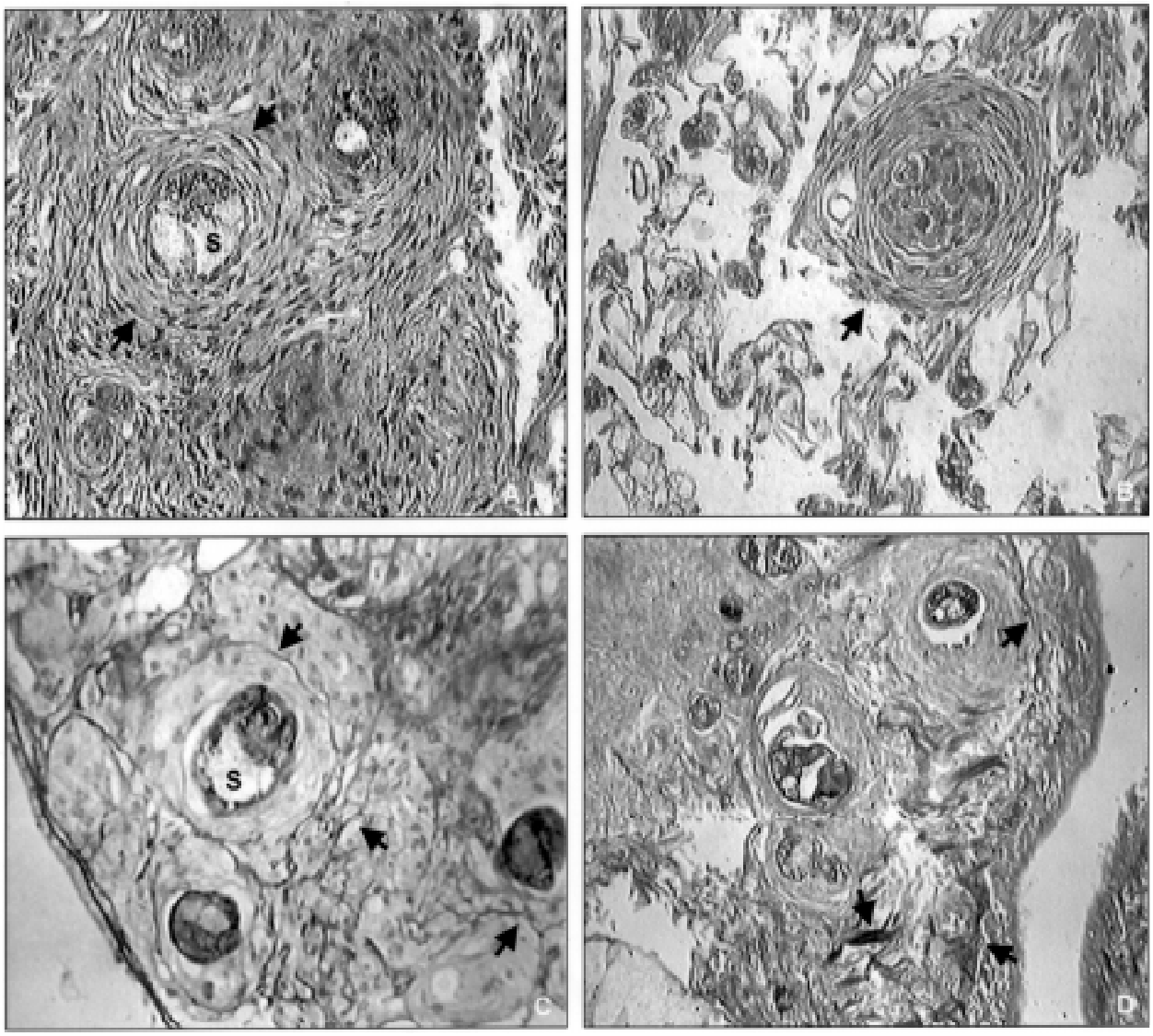

Fig. 2: encapsulating lesions centered by groups of disintegrating sprocysts (S) of *Schistosoma mansoni*. A and B show that concentrically disposed and elongated hemocytes simulating a fibrous granulomatous structure (short arrows); sections stained for elastic fibers (C) and collagen (D) exhibit only a few circular or scattered fibers at the periphery of the encapsulating lesions (short arrows). A, B: Hematoxylin & Eosin, A: 400X and B: 200X; C: Orcein stain for elastic fibers, 400X; D: Sirius-red method for collagen, 200X

sue, such as the foot region and digestive glands, numerous thin and long fibers were seen forming sometimes a cris-crossing pattern. These fibers took a strong red staining with sírius-red and appeared bi-refringent, with striated periodicity, when observed under polarizing light. The color hue varied from red to yellowish. No focal or diffuse imflammatory reaction was observed.

DISCUSSION

Present study was aimed at finding evidences of a primitive participation of the extracellular matrix in defense mechanisms of mollusks. It represents an exercise in comparative pathology, regarding an invertebrate, devoid of lymphocytes, which mounts a defense mechanism by means of a macrophage-like cell that destroys an invading parasite by forming focal encapsulating structures, similar to a vertebrate granuloma, with interactions with humoral factors. (Loker & Bayne 1986, Loker 1994). Such morphological structures are composed by a homogeneous population of cells, with expanded cytoplasmic processes. The presence of cytoplasmic prolongations from numerous cells, appears under the light microscope as containing fibers, sometimes mimicking the process of fibrosis seen in vertebrates. Fibers with staining characteristics of collagen or elastin have been demonstrated in normal snail tissues (Lemos 1999). However, the presence of elements from the extracellular matrix in the granulomatous lesions of *B. glabrata* against *S. mansoni* has been a controversial issue. Although Yoshino (1976) and Krupa et al. (1977) noted that the presence of extracellular fibrils contributed to the formation of the encapsulating lesions, Harris (1975) did not find extracellular elements associated with the molluskan cellular reactive responses. One probable cause for these divergences could be the presence of true collagen and orcein-positive elastic fibers only at the periphery of the lesions, as noted in the present study. Both at light microscopy and ultra-structurally, collagen-like fibers are noted at the proximity of hemocyte accumulations. Since no active connective-cell was visualized and no real accumulation of such fibers could be documented, they are probably pre-existing normal components of the molluskan tissues.

This study was also concerned at identifying the types of hemocytes present in the lesions. Only one cell type could be disclosed by light and electron microscopy at the tissue level. Only the granulocytes could be posi-

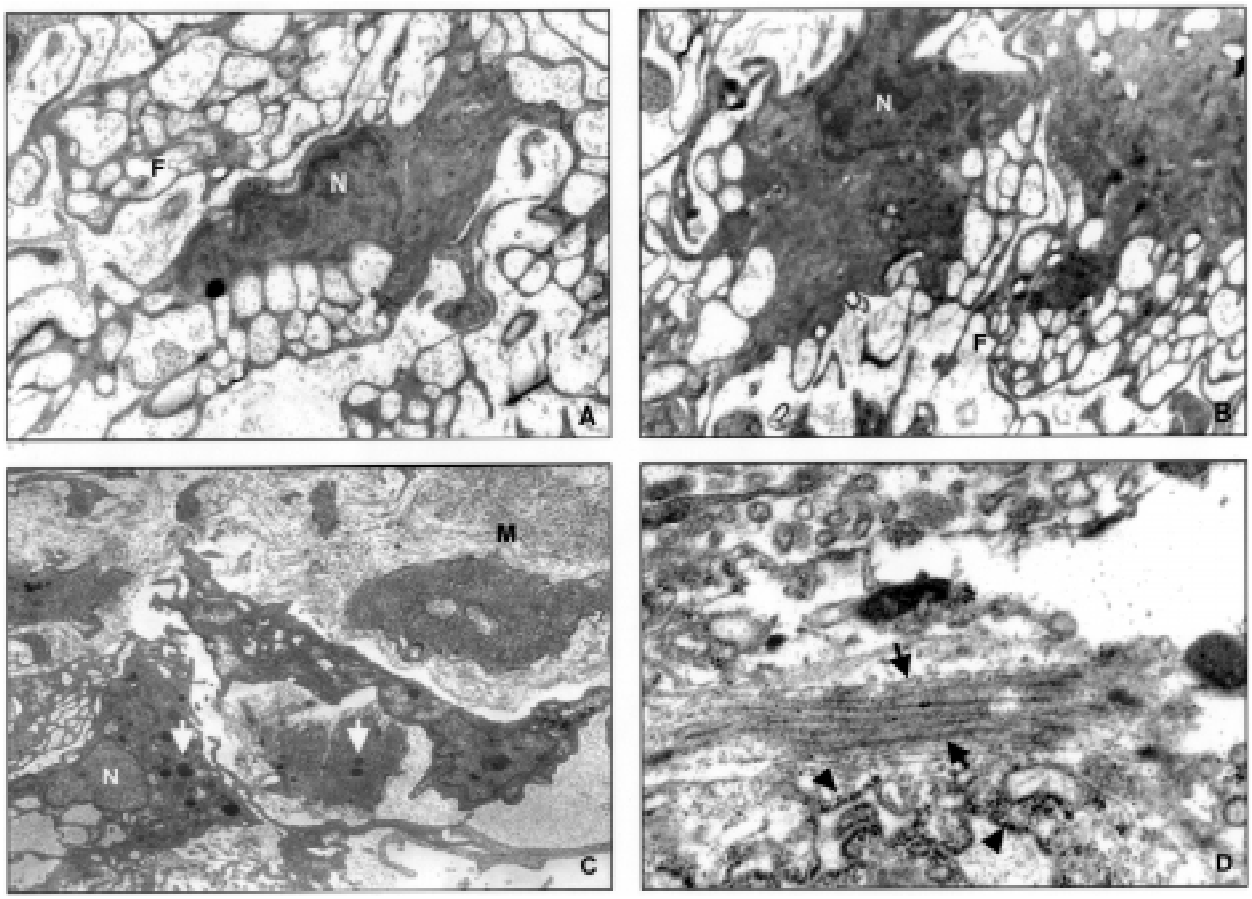

Fig. 3: A and B show hemocytes with their numerous filopodia as part of an encapsulating structure. Besides the filopodia (F), the nucleous (N); C: representative of hemocytes at the periphery of an encapsulating lesion. They appear in the middle of an amorphous and fibrilar extracellular matrix (M). Short arrows are pointing to electron-dense bodies (lysosomes). D: parallels fibrils with periodicity are forming a fiber ate the periphery of an encapsulating lesion. Just below there, a part of a connective cell cytoplasm with dilated endoplasmic reticulum (arrow heads) is shown. Electron microscopy, A, B: 7,000X; C: 3,000X: D: 12,000X

tively identified. The present study and those of Lie and Heyneman (1980) revealed that these cells contain numerous primary and secondary lysosome-like structures, extensive pseudopodia processes, sometimes containing sporocyst in phagolysosomes. However, it was confirmed that two types of hemocytes are present in peripheral hemolymph: granulocytes and hyalinocytes. As a matter of fact, not only the present observations, but many others (Stang-Voss 1970, Sminia 1972, Sminia & Barendsen 1980), clearly shown that hemolymph cells of pulmonate gastropods are a group of morfologically heterogeneous cells. Two kinds of hemocytes are found in fresh hemolymph samples of *B. glabrata.* Probably, the round and spreading cells distinguished in stained preparations are the hyalinocytes and granulocytes described by various authors (Cheng 1975, Yoshino 1976). In peripheral lymph preparations, these cells differ greatly with respect to the development of their organelles (pseudopodia, Golgi bodies, lysosomes) (Barracco et al. 1993).

Collagen is very much conserved in the animal kingdom. The present attempt to measure the concentration of hydroxiproline in molluskan tissues was probably made for the first time and the technique may require better controls. However, no significant differences were noted in normal and *S. mansoni*-infected snails submitted to the biochemicaal determination of hydroxyproline. The data are in keeping with other present findings, which did not reveal any increase of extracellular matrix in *B. glablata,* during its reaction to *S. mansoni* invasion.

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