Histopathological study on susceptible and resistant *Bulinus truncatus* snails to infection with *Schistosoma haematobium*

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In this study, the distribution pattern of *Schistosoma haematobium* miracidia level to host susceptibility/resistance and the basic cellular responses during the parasite development was investigated. Several snail stocks showed a wide spectrum of host reaction to the parasite. A vigorous "resistant-type" cellular response to invading miracidia was seen in the histological sections of non-susceptible snails. In this respect, they were classified as "resistant snails". *Bulinus truncatus* infected with *S. haematobium* exhibited a wide range of histopathological change, suggesting the presence of endogenous factors preventing the immune system of susceptible snails from destroying the developed parasite larvae. Therefore, the mechanism underlying the susceptibility of the snails should be investigated by studying the host-parasite interactions by light and electron microscopy.

Key words: *Bulinus truncatus*, *Schistosoma haematobium*, snails, susceptible, resistance, light microscopy, electron microscopy.

INTRODUCTION

Schistosomiasis is a chronic debilitating disease in tropical regions of Africa, Americas and Asia. The relationships between schistosomiasis and its intermediate hosts, mollusks of the genus *Biomphalaria* and *Bulinus* have been a concern for decades. It is known that the vector mollusk shows different susceptibility against parasite infection whose occurrence depends on the interaction between the forms of trematode larvae and the host defense cell (Oliveira et al., 2010). The genetics of susceptibility of the different strains of snails to *Schistosoma haematobium* infection is complex and likely involves the interaction of several snail and parasite genes (Stothard et al., 2001; Mubila and Rollinson, 2002).

As a vector of *S. haematobium*, the gastropod *Bulinus truncatus* represents the primary model used to investigate the molluscan internal defense system. Hemocytes are the primary effectors of the snail defense system and its resistance lies in the ability of circulating hemocytes to recognize and bind the parasite surface and then undergo a cytotoxic activation, resulting in the effective killing of the parasite (Matricon-Gondran and Letocart, 1999; Bahgat et al., 2002; Martins-Souza et al., 2006). Some gene combinations allow the parasite to develop and proliferate because the snail fails to recognize it as foreign. In this combination, the parasite is recognized and phagocytized in one to a few days (Lie et al., 1987), or the parasite fails to develop because the host constitutes an unsuitable medium (Lie et al., 1983).

The fate of sporocysts of *S. haematobium* in non-susceptible *B. truncatus* has been studied by numerous investigators. Early study by Lie et al. (1977) reported that manifestations of non-susceptibility range from rapid, hemocyte-mediated destruction of sporocysts, a phenomenon termed resistance, and one that is believed to represent an immunological response (Adema et al., 1997) to arrested or delayed development (Lewis et al., 1993).
MATERIALS AND METHODS

Snails and exposure to infection

Laboratory-reared, adults B. truncatus snails (6 -10 mm shell height; age, 6 - 8 weeks) and miracidia of S. haematobium were supplied by the Schistosome Biological Supply Center [SBSC, Theodor Bilharz Research Institute (TBRI), Giza, Egypt]. Snails were maintained as described by Liang et al. (1987). B. truncatus snails were exposed individually for 3 h in 1 ml dechlorinated water to 5 - 10 freshly hatched S. haematobium miracidia. Exposed snails were examined daily for cercarial shedding by exposing them to fluorescent light for 2 h from the first week up to 6 weeks. Snails were classified into susceptible and resistant according to Lewis et al. (1993).

Light microscopy

To study parasite development, at each time of testing, 3 - 5 snails from susceptible and resistant strains were fixed in Bouin’s fixative for at least 24 h and then placed in gradually increasing concentrations of ethanol. Hematoxylin-eosin-stained 5-µM sections were examined microscopically for the histological condition of larval trematodes, as categorized by Borges et al. (1998). Histological analysis was performed on about 38 and 45 susceptible and resistant snails, respectively.

Electron microscopy

Snails aseptically removed from the shell were immediately fixed by immersion in iced 2.5% glutaraldehyde in 0.2 M phosphate buffered (pH 7.2) for 2 h and post-fixed in 0.05 M sodium cacodylate buffer and 1% osmium tetroxide in the same buffer and subsequently dehydrated in acetone for embedding in Araldine. Ultrathin sections from selected blocks were obtained with a Reichert OM-43 Ultratome, double-stained with lead citrate and uranyl acetate and examined with a Jeol 100 C Electron Microscope at 50 kV. Semithin sections approximately 1 - 2 µM in thickness, stained with alkaline toluidine blue, were used for selecting the area of interest.

RESULTS

A total number of 150 snails from both susceptible (75) and resistant (75) groups, respectively, were used. Survival rate of snails after exposure to S. haematobium miracidia was found to be quite similar in both groups (80 - 81.3%). The data depicted in Figure 1 indicated that normal infection was observed in 53.3 and 9.3% in the susceptible and resistant snail groups, respectively, at 1 - 2 weeks post exposure (WPE). Moreover, 1.8 and 3.3% of susceptible and resistant snails, respectively, developed such retarded infection signs with the development of foot-sporocyst before the 3 WPE. Meanwhile, retarded infection signs were observed in 0.78 and 9.7% of susceptible and resistant snails, respectively, but with the development of foot-sporocyst in more than 3 - 5 WPE. The data also shows that 46.7 and 90.7% of the susceptible and resistant snails, respectively, did not show any sign of infection at 7 WPE.

Moreover, cellular reaction to the sporocysts varied with sporocysts location and the length of infection. At 1 WPE, most of the sporocysts contained one or more germinal cells with nucleoli and, therefore, were judged to be viable. Approximately 8 - 12% of the sporocysts were elongated, showing transverse constrictions and were categorized as normal (Figure 2A); those that showed no elongation or folding were categorized as retarded. Some sporocysts were surrounded by several layers of flattened hemocytes (Figure 2B), but many others were free of encapsulation. All remaining sporocysts at 3 - 4 WPE were categorized as dead, that is degenerated (all germinal cells lacking nucleoli) or destroyed (disintegrated and undergoing phagocytosis) (Figure 2C). At 5 WPE, all sporocysts were dead. Most degenerated sporocysts were compact, round, and contained scattered pyknotic nuclei in a vacuolated eosinophilic matrix (Figure 2D). In addition, other bodies lacking nuclei were seen at these two subsequent time periods. These were usually round to oval, homogenously eosinophilic except for vacuolated areas, and were surrounded by a clear space (Figure 2E).

The histological study showed that the final site of infection, developmental and growth dynamics were similar with previous reports. Although encapsulation of sporocysts never occurred in susceptible snails, hemocyte aggregations could sometimes be observed in the proximity of well developed sporocysts (Figure 3A). Figure 3B shows
Figure 2. Histological sections of *B. truncatus* exposed to *S. haematobium*. (A) Normal sporocyst in head-foot at 1 week post exposure (WPE); note absence of hemocytic response. (B) Sporocysts (arrow) surrounded by hemocytes (arrow head) at 4 WPE in the tentacles. (C) Degenerating sporocyst (arrow) and large capsule formation (arrow head) in the tentacles at 4 WPE. (D) Destroyed sporocysts in the tentacles of susceptible *B. truncatus* exposed to *S. haematobium* at 5 WPE; note eosinophilic fragments (arrows) undergoing phagocytosis by hemocytes (arrow head). (E) Amorphous sporocyst in the pericardial cavity of the heart at 5 WPE. Sporocysts consisted of vacuolated eosinophilic matrix (arrow) (x160).

Figure 3. (A) Degenerating (collapsed) sporocyst in the mantle of susceptible *B. truncatus* exposed to *S. haematobium* at 3 WPE; note narrow, darkly stained germinal cells and surrounded by several layers of hemocyte. (B) Degenerating (rounded) sporocyst in the kidney of susceptible *B. truncatus* exposed to *S. haematobium* at 4 WPE; note eosinophilic masses (arrow), pyknotic nuclei surrounded by flattened hemocytes. (C) A numerous multiplying sporocysts (arrow) appeared within of the heart of resistant *B. truncatus* exposed to *S. haematobium* at 2 WPE; note the absence of any tissue reaction. (D) An encapsulated focal reaction (arrow) in the mantle collar of resistant *B. truncatus* exposed to *S. haematobium* at 3 WPE. (E) A sporocyst (arrows) in the anterior cephalopodal sinus of resistant *B. truncatus* exposed to *S. haematobium* at 4 WPE; note individual hemocytes (arrow head) adhering to the parasite surface. (F) Destroyed sporocysts (arrow) in the head-foot of susceptible *B. truncatus* exposed to *S. haematobium* at 6 WPE (x160).
normal developing sporocysts in susceptible snails at 4 WPE for comparison with sporocysts in resistant snails. In the resistant snails, miracidia were able to transform into sporocysts, to migrate and reach the heart area by the end of the 2 WPE (Figure 3C). Although the migration pattern was overall similar to that of susceptible snails, differences could be observed regarding sporocysts final site of infection (Figure 3C). This cellular response continued to increase and, after 3 - 4 WPE, resulted in the encapsulation of the sporocysts settled in the ventricle and aorta. Sporocysts tegumental destruction was observed and hemocytes were infiltrating the sporocyst tissues. In some cases, a clear space was still observed between the sporocyst tegument and the capsule (Figure 3D), although individual hemocytes were present at the surface of the parasites (Figure 3E).

Interestingly, this encapsulation process was not observed for sporocysts settled in the pericardial cavity. Despite this absence of encapsulation, the development of the sporocysts settled in the digestive gland was not normal as compared with the development of sporocysts in susceptible snails. After 4 WPE, sporocysts settled in the kidney, mantle collar, ventricle and the aorta were clearly degraded. In addition to the large number of hemocytes encapsulating sporocysts, numerous hemocyte aggregations were observed in the heart area, such as the pericardial cavity. After 5 WPE, sporocysts were fully degraded and hardly identifiable within capsules. In some cases, degenerate sporocyst materials were observed (Figure 3F). The capsule was characterized by numerous dead or degenerating hemocytes in the center next to the dead sporocysts surrounded by live hemocytes. The external surface of the sporocysts is increased by irregular knobs and short ramified projections. The 1 to 4 µM thick tegument may have deep fissures, or numerous deeply channels. The tegument is rich in vesicles and vacuoles which vary in size and shape and have more or less electron-dense contents. Mitochondria, myelin-like bodies and electron dense spherical bodies are common in the tegument. The spherical bodies are formed in the tegumented cell bodies that occur below the basal lamina and are in common with the syncytial tegument through thin cytoplasmic bridges (Figure 4A). Poorly developed and sparsely described muscle fibers occur below the basal lamina (Figure 4B). The sub-tegumental layer also contains different types of parenchymatous cells, some of which are rich in lipid droplets and glycogen patches (Figure 4C). Many sporocysts occur close to tubules of the digestive gland. The tegument of the sporocyst and the basal lamina of the hemocyte-producing organ (HPO) are separated by connective tissue cells or muscle fibers (Figure 4D).

Aggregating hemocytes form a compact capsule around the damaged sporocysts. The peripheral flattened hemocytes are very similar to the flattened cells that surround living sporocysts. Some cells contain small spherical, lysosome-like vesicles and high concentrations of supposed glycogen particles (Figure 4E). The innermost cells of the capsule act as phagocytes. Phagocytosis of parasite tissue is followed by formation of residual bodies of indigestible material in cells in the center of the capsule (Figure 4F). As some of these cells die a dense core of residual bodies is formed. It is likely that some of the peripheral cells of the capsule become amoebocytic and migrate to other areas of the snails (Figure 4G). The cells containing the residual bodies may migrate from the capsule center to the peripheral layer. However, most cells in the capsule remain and a homogenous tissue replaces the removed parasite (Figure 4H). This granuloma formation is composed of large rounded cells containing lysosome-like vesicles and small spherical vesicles (Figure 4I).

**DISCUSSION**

In the present study, we preferred to select laboratory-reared snails from different stocks to investigate resistance in *B. truncatus* to infection with *S. haematobium*. This selection process allows adult snails to self-fertilize, exposes the juvenile progeny to infection and ensures the isolation of the susceptible/resistant (positive/negative) snails. Through different analysis, some snail stocks showed normal developing infection, although some snails of each stock showed no signs of infection and/or delayed infection with or without foot sporocysts development according to the classification of Kristensen and Christensen (1989) and Sesen and Yildirim (1993). In snails that develop foot-sporocysts, it seems that the genetics of this phenotype probably involve multiple factors expressed in variable quantitative doses in the snail (Özcel et al., 1996). Although retarded schistosome infections have been discussed by other researchers, Sesen (2004) proposed that an additional category of snail/parasite interaction must be added to the four general classification described in details by Mukaratirwa et al. (1998).

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; Borges and Andrade, 2003). However, the presence of elements from the extracellular matrix in the granulomatous lesions of *Biomphalaria glabrata* against *Schistosoma 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 molluscan cellular reactive responses. One probable cause of this divergence could be the presence of true collagen and orcein-positive elastic fibers only at the periphery of the lesions as noted recently by Borges and Andrade (2003). Both at light and electron microscopy, collagen-like fibers are noted at the proximity of hemocytes accumulations. Since no active connective-cell was visualized and no real accumulation of such fibers
Figure 4. (A) An electron micrograph of a section through the tegument of sporocyst from *B. truncatus* exposed to *S. haematobium*. Note the large nucleus (N) with its prominent nucleolus, large secretory granules (arrow), Golgi complex (G), mitochondria (m) and granular endoplasmic reticulum (ER). (B) The tegument is moderately electron dense and contains mitochondria (m) and membrane-bound granules. Adjacent cells (arrow) are ventricular smooth muscle. (C) Intercellular space (arrow heads) between newly aggregated cells; sporocyst tegument is swollen with loss of mitochondria (m) and erosion of membrane-bound granules (arrow). (D) Hemocytes extending homogenous pseudopodia (arrow) toward lipid-containing tegument of sporocysts. (E) Debris-laden hemocytes surrounding degenerating sporocysts (arrow). (F) The outer part consists of lightly packed flattened hemocytes (arrows); remains of dead sporocysts are (arrow head) seen in the center of the capsule. (G) Note portion of hemocytes in cell aggregation in the ventricle. Immature features include abundant free ribosomes (R). (H) Cells containing residual bodies (arrow) from the center of the capsule. (I) Rounded cells from the center of the granuloma formation (x1200).

could be documented in the present study, they are probably pre-existing normal component of the molluscan tissues as suggested by Imbert-Establet et al. (1992), Vuong et al. (1996) and Botros et al. (2008).

Encapsulation and subsequent destruction of helminths by molluscan hemocytes is well known (Mubila and Rollinson, 2002; Remy and Arouna, 2005). The speed and severity of encapsulation responses against trematode larvae have been hypothesized to reflect the degree of host resistance, with rapid encapsulation and destruction being interpreted as evidence of strong resistance (Kirinoki et al., 2000; Sasaki et al., 2003; Azevedo et al., 2006). Accordingly, resistance in *B. truncatus* has not been deemed strong because the hemocytic reaction is unimpressive and dead parasites persist for some time. Since the parasites seem to be recognized and
encapsulated but not readily phagocytized, these snails may be useful in determining if encapsulation and phagocytosis require specific genetic activators as reviewed by Richards (1975). This fact must be considered in discussing the biological control of *S. haematobium* by the introduction of refractory snails into endemic areas.

Among non-susceptible stocks of *B. truncatus* to *S. haematobium* in the present study, there is a range of host tissue response and parasite deterioration at several intervals following infection. Meanwhile, we do not know whether the range of the deterioration of *S. haematobium* sporocysts observed is the result of a differential host response to a homogenous parasite population or whether it reflects the range in parasite diversity as suggested by Sullivan et al. (2004). In the present study, a snail stock was categorized as non-susceptible. Although a few snails in this stock became infected, a vigorous “resistant-type” cellular response to invading miracidia was seen in histological section as that of non-susceptible snails. In this respect, they resemble the other resistant snails stocks (Lie et al., 1987; Lewis et al., 1993; Cooper et al., 1994; Cousin et al., 1995), and are classified in our study as “resistant snails” notwithstanding the low percentage of susceptible reactions in this stock.

The present light microscopic observations of the interaction between sporocysts of *S. haematobium* and *B. truncatus* snail tissue generally agree with similar studies of sporocysts in natural or susceptible hosts (Sminia and Barendsden, 1980; Arfaa et al., 1989; Lemos, 1999; Borges and Andrade, 2003). Electron microscope studies of the relation between sporocysts and snail cells are limited and possibly rare. The general morphology of the body wall of the sporocysts of *S. haematobium* does not differ essentially from that of most sporocysts or radiae described in the ultrastructural level (Kirimoki et al., 2000; Sasaki et al., 2003; Remy and Arouna, 2005). However, some sporocysts have been described as possessing an external nucleated layer outside the tegument. This external layer is often mentioned as the “Paletot”. Such two-layered sporocysts have been described at the ultrastructural level (Hussein et al., 2005; Sasaki et al., 2003; Kirinoki et al., 2000). However, some sporocysts have a nucleated cell layer of any sporocysts or redia when these occur in the molluscan tissue. The cell layer (primitive epithelium) which surrounds developing daughter sporocysts disappears before these leave the mother sporocysts (Meuleman et al., 1980; Southgate et al., 1989; Joubert et al., 1991; Souza et al., 1995; Lemos, 1999).

Host reactions resulting in the formation of few layered or loosely packed host capsule around daughter sporocysts in susceptible host have been described at the ultrastructural level by Krupa et al. (1977). These host responses may, similarly to the layers of flattened hemocytes around the sporocyst of *B. truncatus*, be regarded as an attempt by the host to wall off the parasite. The tegument of the sporocyst is the sole organ for uptake of nutrients. Absorption by the external surface of sporocysts may be enhanced by the presence of structural modification such as microvilli, folds, ridges and invaginations (Zdarska and Soboleva, 1982) that amplify the surface area of the tegument. The extensive amplification in surface area of trematode may be correlated with their capacity to actively transport among other things (Uglem and Lee, 1985).

**Conclusion**

The cellular response observed in *S. haematobium* resistant stock of *B. truncatus* support the notion that a rapid destruction of the mother sporocysts depends on hemocyte capability to adhere to and encapsulate the parasite. However, observations of a related development and degeneration of un-encapsulated or partially encapsulated sporocysts raise the question of the possible role of humoral factor in the resistance to *S. haematobium*. Therefore, further studies are needed to determine the impact *S. haematobium* infection on morphological and functional properties of *B. truncatus* hemocytes.

**REFERENCES**


