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A COMPARATIVE STUDY OF HAEMOCYTES FROM RESISTANT AND SUSCEPTIBLE *LYMNAEA NATALENSIS* SNAILS EXPOSED *TO FASCIOLA GIGANTICA* MIRACIDIA

By

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Abstract

Effect of infection with *Fasciola gigantica* on total and differential haemocytes count of resistant and susceptible *Lymnaea natalensis* snails were studied.

Exposure of *L. natalensis* resistant and susceptible strains to *F. gigantica* on miracidia caused gradual increase in the number of circulating haemocytes at the same time of exposure. In susceptible strain, the increase in the number of circulating haemocytes became significant at the second week post exposure being 2560 cell/ml ($p<0.05$) in comparison with control group being 1760 cell/ml. While in resistant strains, the increase became significant at second, third and sixth weeks post exposure being 2660 cell/ml, 2500 cell/ml and 2360 cell/ml respectively,then a gradual decrease occurred. Examination of haemocytes obtained from *L. natalensis* snails revealed that haemolymph contained three morphological types of haemocytes, designated as round small, round large (hyalinoctyes) and granulocytes spreading. Their average percentage was 12.3±5.5%, 81.0±4.6% and 6.7±2.1% of total cells respectively. Data indicated that by the second day post exposure infected snails had significantly higher percentage of granulocytes than controls.

Key words: *Fasciola gigantica*, *Lymnaea natalensis*, Circulating haemocytes, Susceptible snails, Resistant snails.

Introduction

All living species have innate internal defense system and the mollusks also, are therefore not defenseless against parasitic invaders or pathogens. They possess an internal defense system which is different from immune system of humans and higher vertebrates and this system comprises both cellular and humoral elements. The body cavity of fresh water snails (mollusca) is filled with haemolymph which contains dissolved hemoproteins and several kinds of blood cells referred as haemocytes which represent the primary mediators of cellular defense reactions and play an important role in recognition and elimination of invading foreign bodies through phagocytosis, encapsulation and release of cytotoxic mediators (Van Der Knaapand Locker, 1990; Bayne, 1991).

Once a trematode parasite has penetrated the snail host, it has to avoid the activities of the host's internal defense system. An important strategy of immuno-evasion employed by a compatible parasite is to suppress the activity of the host's internal defense system (Van der Knaap*et al,* 1987; Hahn *et al*, 2001). Phagocytosis is a well-known internal immune defense mechanism in all animals. Phagocytosis is considered to be the primary clearance mechanisms in gastropods (Furuta and Yamaguchi, 2001).

Sminia (1972) reported that phagocytosis of foreign particles by amoebocytes involves the pseudopodia spreading to engulf the particles with invagination of the cell membrane, the foreign particles are attached by hydrolytic enzymes (acid phosphatase) and also demonstrated the peroxidases in the lysosomal system of *Lymnaea stagnalis* with bactericidal, viricidal and fungicidal functions similar to that of mammalian leucocytes.

Barraco*et al*. (1993) distinguished two types of haemocytes in *Biomphalaria tenagophyla*; they are hyalinocytes (large nucleus

and basophilic cytoplasm) and granulocytes (large cells with small nucleus and high cytoplasmic content), While El-Sayed *et al*. (2011) revealed that the presence of three morphologically distinct types of haemocytes in the haemolymph of *Biomphalaria alexandrina* namely: hyalinocytes, amoebocyte and granulocyte. Granulocytes can be also classified into two groups named as few granular and heavy granular granulocytes.

Some authors stated that the haemocytes represent different stages of growth and development of a single cell type, from young cell (round) to mature cell (spreading) (Sminia*et al*, 1979; Amen *et al*, 1992). Also, Pan (1958) described one cell type, the amoebocytes, but he recognized that the amoebocyte might further be differentiated into more than this single type. Other authors considered these categories of haemocytes to be different types (Yoshino and Granath, 1985; Loker and Bayne, 1986).

The present study focused on the possible correlation between number and composition of haemocytes from resistant and susceptible *Lymnaea natalensis* snails exposed to *Fasciola gigantica* miracidia.

Materials and Methods

The experimental snails used were adult *Lymnaea natalensis*(shell height ranged between 3-5 mm). The snails were obtained from Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute (TBRI), Egypt.

Snails were allowed to lay egg masses on small foam pieces placing on the water surface of the aquaria in dechlorinated tap water under laboratory conditions according to the method described by El-Emam and Ebeid (1989). The egg masses of adult *Lymnaea natalensis* snails were collected and hatching snails were transferred to another aquaria where they feeding on *nostoc muscorum* algae, chalk as a calcium supplement and aseptic soil (Taylor and Mozley, 1948; Youssef *et al*, 1999) until they reach to 3-5 mm shell height then feeding on lettuce leaves and tetramin (fish food). Aseptic soil and chalk were added to the aquaria until they were used for study purposes. Water in the aquaria was changed weekly, a photoperiodicity of 12hr. light/ 12 hr. dark and water temp. of 23±1°C.

Snails infection: Adult *Fasciola gigantica* worms were obtained from condemned livers of cattle and sheep. The infected liver was cut across and squeezed to recover flukes and eggs from the open end of the biliary duct. Adult worms were incubated at 37 °C for two hours. Large number of eggs was collected from adult worms. The remaining eggs in the worms were obtained by dissecting worms under microscope. The eggs were put in numbers of Petri-dishes containing dechlorinated tap water at 26°C for about 14 days. Miracidia of *Fasciola gigantica*were hatched under illumination, snails (3-5mm shell height) were exposed individually to 8-10 miracidia / snail within 30 minutes from hatching in glass vials with 2 ml dechlorinated tap water, under florescent light at temperature (25±1°C). After 24 hours, the snails were transferred to standard aquaria as described above. After 38 days exposed snails were examined for cercariae-shedding by placing snails individually in a petri-dish (3 cm) and 5 ml dechlorinated tap water with a sheet of plastic. Shedding of cercariae was allowed for 72 hr. and no food was provided, metacercariae were found just beneath the water surface on the plastic sheet.

Snails were classified into susceptible and resistant according to the classification of Lewis *et al.* (1993). Susceptible and resistant snails were selectively isolated and allowed to interbreed in separate aquaria. Egg masses from both groups were collected until the second generation. Juvenile snails resulting from the third generation were subsequently used in the present study. Resistance was histologically demonstrated by the presence of haemocytes surrounding the miracidium 24 h. after infection (Gutierrez *et al,* 2003).

Collection of haemolymph of *L. natalensis* snails from different groups was performed at different weeks post exposure to *F. gigantica* miracidia as described by Negm*et al*. (1995). Water adhering to the snail was removed and the head foot was cleaned with tissue paper. By touching the foot with the point of the micropipette, the snail was forced to retract deeply into its shell and extruded haemolymph. Thus, about 30µl of haemolymph were obtained from each individual snail.

For total haemocytes count, 10µl haemolymph were individually collected from at least 5-10 snails/group after different intervals (2 and 4 days and 1,2,3,6 and 8 weeks) post exposure to miracidia, another group was used as control. The number of cells in both experimental and control groups was counted using a Bürker-Turk hemocytometer.

Haemocytes monolayers were prepared by placing 10 µl of haemolymph on a glass slide and haemocytes were allowed to adhere the glass in a moist chamber for 15 min. at room temperature, then rinsed with snail Wringer buffer/10 mMCa^{++} (SR) pH 7.3, and incubated in the same buffer for 10 min. Haemocytes were dehydrated with methanol for 5 min. at room temperature, rinsed several times with SR, stained with 10% Giemsa stain (Aldrich) in buffered distilled water (0.021 MNa_2 HPO_4/0.015 MKH_2PO_4), pH 702 for 30 min. Differential hemocyte counts were recorded in both experimental and control groups and the mean ± standard deviation for each hemocyte population was calculated.

Statistical analysis: Values of total and differential hemocyte numbers were expressed as mean ± S.D. and the obtained data were statistically analyzed using "t" test and analysis of variance (ANOVA) to determine the significant differences in means between the control and experimental groups, using the statistical program for windows (SAS, 2000).

Results

L. natalensis resistant and susceptible strains did not exhibit a significantly different number of circulating haemocytes (at $p<0.05$) at the same time of exposure. Data (Tab. 1) showed that exposure of susceptible snails to *F. gigantica* miracidia caused gradual increase in the number of circulating haemocytes, the increase in number of circulating haemocytes was not statistically significant from the second day post exposure until the first week post exposure, but became highly significant at the second week post exposure, then a gradual decrease occurred till the eighth week post exposure where the total number of haemocytes nearly reached the control level.

The exposure of resistant snails to *F. gigantica* miracidia caused gradual increase in the number of circulating haemocytes, the increase in number of circulating haemocytes was not statistically significant from the second day post exposure until the first week post exposure, but became highly significant at the second and third weeks post exposure, then a gradual decrease occurred. Also,the increase in the number of haemocytes in resistant strains was more than in susceptible one. This may be due to that, haemocytes are considered the primary effectors of snail immune response to parasitic infection.

Examination of haemocytes obtain from *L. natalensis* snails revealed that haemolymph contained three morphologically distinct types of haemocytes, designated as round small, round large (hylinoctyes) and granulocytes "spreading" Fig. (1A,B). The round small cells (8-10µm diameter) showed a small nucleus occupying the center of the cell and cytoplasmic layer occupying the peripheral position around nucleus. Their average percentage was 12.3±5.5% of total cells. The round large cells (12-15µm diameter), had a central spherical large nucleus occupying most of the cell, their average percentage was 81.0±4.6% of total cells; these cells were not adherent on glass.

Granulocytes or spreading cells (20-30µm diameter) appeared as irregular large cells producing long filamentous pseudopods, characterized by many cytoplasmic granules, and had rounded nucleus and large round cytoplasm. Their name denotes "spreading" that are active cells possessing number of pseudopods taking role of phagocytosis. The number of spreading cells was very few in unexposed, their average percentage was 6.7±2.1% of total cells.

During the first day post exposure (PE) the percentage of hemocyte categories were unaffected. However, Data revealed that by the second day post exposure infected snails were significantly had higher percentage of granulocytes till 6 WPE than in control groups(Tab 2, Fig. 2). This was accompanied by significant decrease in round small and round large haemocytes (Tabs. 3 & 4; Figs. 3 & 4). This gave an indication that immature undifferentiated cells eventually differentiate into spreading granulocytes.

Discussion

Haemocytes are considered as the primary effectors of snail immune response to parasite infection, several studies have focused on the possible correlation between the hemocyte number or compostition and *Lymnaea* spp. immune reactivity. Previous studies showed that *L. natalensis*is more susceptible to *F. gigantic* infection as compared with *L. glabra* and *L. trunculata* (Bouix-Bussone*t al,* 1984), and that *L. natalensis* also possess fewer circulating haemocytes than do other *Lymnaea* spp. (Sminia and Barendsen, 1980; Van der knap *et al,* 1987; Pokora, 1994; Kofta, 1997). In addition, experiments using *L. natalensis* with various hemocyte loads and various degree of susceptibility to *F. gigantica* strongly suggested that the number of circulating haemocytes influences the snail vulnerability to infection with *F. gigantica* (Helal and Abdel-Maksoud, 1999). However, in the present study, resistance to *F. gigantica* is not correlated with the number of circulating haemocytes as both the susceptible and resistant strains exhibit similar number of circulating haemocytes.

In this study three main types of haemocytes could be detected in the haemolymph of the snail *L. natalensis* using light microscopy. These are small round, large round and granulocytes. This agrees with the findings of Sminia (1972), Noda and Loker (1989), Vander Knaap and Loker (1990) and Sharf El-Din (2003) while Pan (1958) observed only one cell type, the amoebocyte. But cheng and Auld (1977); Ottaviani and Franchini (1988) found two types, hyalinocytes and granulocytes in *B. glabrata* & *Planorbarius corneus* respectively. Neanwhile Jokey *et al*. (1985) characterized four types among granulocytes of *B. glabrata* according to their cell receptor. El-Sayed *et al*. (2011) found three types of haemocytes distributed in the haemolymph of *Biomphalaria alexandrina* and *Biomphalaria glabrata* snails. These cells are distinguished according to their shape, size and number and are named granulocytes, hyalinocytes and amoebocytes.

The internal defense system of snails consists of both cellular and humoral components. Circulating haemocytes are the principle line of cellular defense. They can be bound to and kill trematode larva by phagocytosing the syncytial tegument or releasing cytotoxic compounds or both (Yoshino and Vasta, 1996). The susceptibility of fresh water snails of genus *Biomphalaria* to infection by *S. mansoni* is linked to the haemocytes present in the haemolymph (Serrano *et al*, 2002). These cells present morphological and biochemical heterogeneity that make their characterization based on simple criteria difficult (Loker*et al*, 1982). The present work showed that *L. natalensis* is resistant to *F. gigantica* and also showed variability in the type of circulating haemocytes. Other authors have pointed out that the number of haemocytes in the haemolymph is influenced by the snail's age, physiological condition and by the method of obtaining (Yoshino and Vasta, 1996; Granath and

Yoshino,1984). Haemocytes are not easily stained and a better contrast is obtained with May- GrünwaldGiemsa. Four types of haemocytes were reported in *Biomphalaria* isolates (Delgado *et al*, 2001). The adherence to glass assays showed that haemocytes had engulfing property and the ability to encapsulate *S. mansoni* miracidia in 1 hr. of contact. Results of similar studies suggest that detection of role of haemocytes, although likely, may require different assays, possibly of a more prolonged nature (Sapp and Loker, 2000). Other studies conclude that the encapsulation process and the production of toxic radicals against the parasite define resistance (van der Knaap and Loker, 1990). So, the *Biomphalaria* hemocyte interaction with *Schistosoma* miracidium could be of concern for evaluation of cellular immunity of this snail against infections and could affect schistosomiasis control programs.

The expectation that changes involving the haemocytes within the *L. natalensis* tissues would have a repercussion on the peripheral haemolymph did not come true. Although the snails used were very susceptible to *F. gigantic* infection, large areas of hemocyte infiltrations did occur in some specimens, but variations in hemocyte counting and in their signs of increased phagocytic activity were always scarce. Probably the two compartments are independent, and nothing similar to a vertebrate leukocytosis is to be expected during the occurrence of inflammatory-like changes in the snail tissues.

Conclusion

The outcome results showed that resistance was not correlated with the number of circulating hemocytes as both the highly susceptible and highly resistant strains exhibit similar number of circulating hemocytes at the same time of infection. The infection with *Fasciola gigantica* caused a significant increase in the number of granulocytes, this was accompanied by significant decrease in round small and round large hemocytes. This gives an indication that immature undifferentiated cells eventually differentiate into spreading granulocytes.

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Table 1: Total haemocytes count/ml of *L. natalensis* susceptible and resistant exposed to *F. gigantica* miracidia.

	Control	2d	4d	1w	2w	3w	6w	8w
Susceptible	1760± 704.3	1940± 377.7	2020± 345.8	2200± 377.1	2560± 323.9**	2220± 289.8	1820± 404.9	1780± 537.1
Resistant	1760± 704.3	2060± 422.2	2180± 382.3	2220± 446.7	2660± 481.2**	2500± 368.2**	2360± 323.9*	2120± 434.1

Data expressed as mean X 10^3 ± S.D.*= significant at p<0.05, **= highly significant at p<0.01

Table 2: Percentages of granulocytes of *L. natalensis* susceptible and resistant exposed to *F. gigantica* miracidia.

	Susceptible	Resistant
Control	6.7±2.1	6.7±2.1
2d	21.3±8.0**	15.0±1.0*
4d	18.7±4.7*	14.3±3.5*
1w	26.7±5.1**	20.3±2.1**
2w	28.0±4.6**	18.0±2.0*
3w	17.7±3.5*	15.0±4.0*
6w	20.0±4.0*	12.0±2.6
8w	10.0±3.6	11.0±2.0

*= significant at p<0.05, **= highly significant at p<0.01

Table 3: Round small haemocytes% of *L .natalensis* susceptible and resistant exposed to *F. gigantica* miracidia.

	Susceptible	Resistant
Control	12.3±5.5	12.3±5.5
2d	15.7±0.6	17.0±6.0
4d	6.3±2.1*	7.3±1.5*
1w	4.7±1.5**	6.0±1.0*
2w	3.3±0.6**	4.7±2.1**
3w	7.3±2.1*	6.0±2.6*
6w	5.3±2.3*	8.3±0.6*
8w	9.3±4.2	9.3±2.5

*= significant at p<0.05, **= highly significant at p<0.01

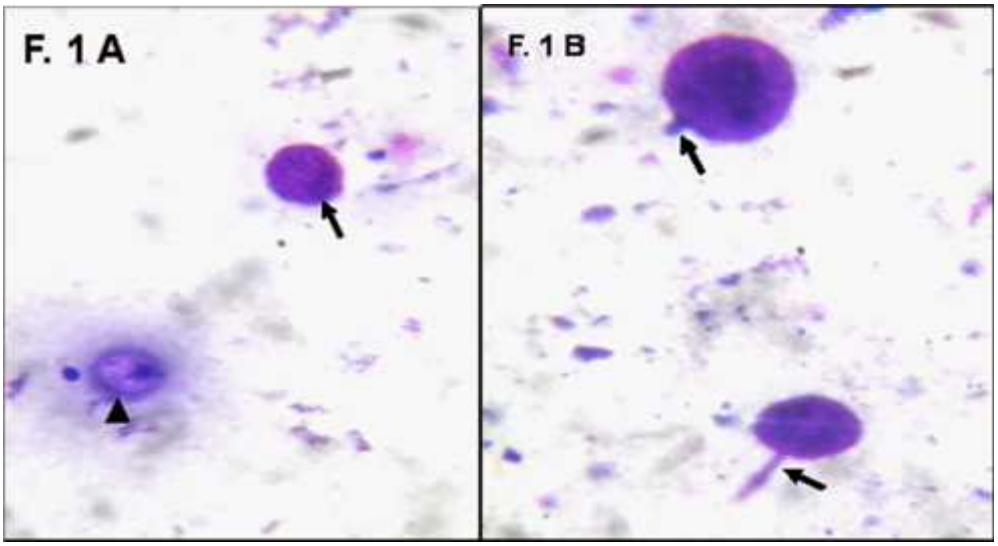
Table 4: Round large haemocytes% of *L.natalensis* susceptible and resistant exposed to *F. gigantica* miracidia.

	Susceptible	Resistant
Control	81.0±4.6	81.0±4.6
2d	63.0±7.5**	68.0±5.6**
4d	75.0±3.0*	78.4±2.9
1w	68.7±5.5**	73.7±1.5*
2w	68.7±5.0**	77.3±3.2
3w	75.0±5.6*	79.0±2.6
6w	74.7±6.1*	79.7±2.1
8w	80.7±5.5	79.7±4.0

*= significant at p<0.05, **= highly significant at p<0.01

Explanation of figures

Fig. 1 A: Light photomicrograph of haemocytes from control *Lymnaea natalensis* snail showing round small (arrow head) and round large (arrow) hemocyte types.. B: haemocytes from control *L. natalensis* showing granulocytes. Note: granulocytes forming long filamentous pseudopods (arrows). X1000.
Fig. 2: Granulocytes% of *L. natalensis* susceptible and resistant snails exposed to *F. gigantica* miracidia.
Fig. 3: Small round haemocytes% of *L. natalensis* susceptible and resistant exposed to *F. gigantica* miracidia.
Fig. 4: Large round haemocytes% of *L. natalensis* susceptible and resistant exposed to *F. gigantica* miracidia.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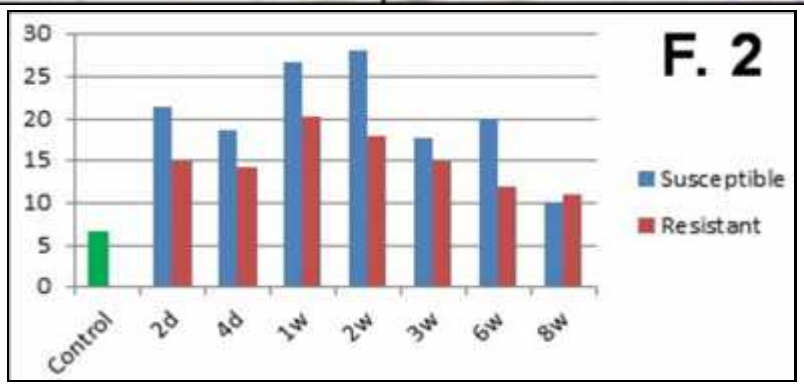
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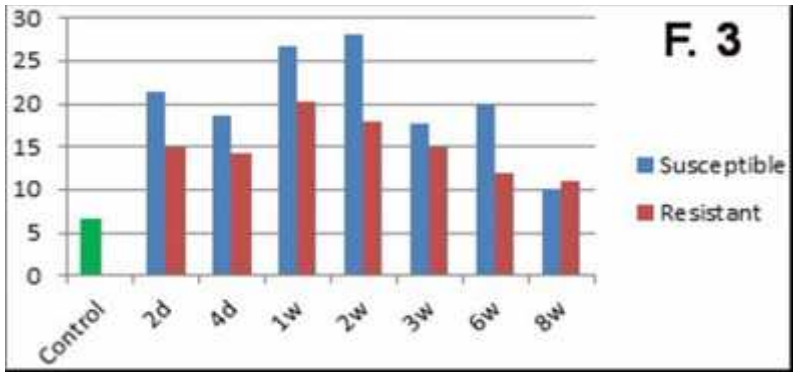
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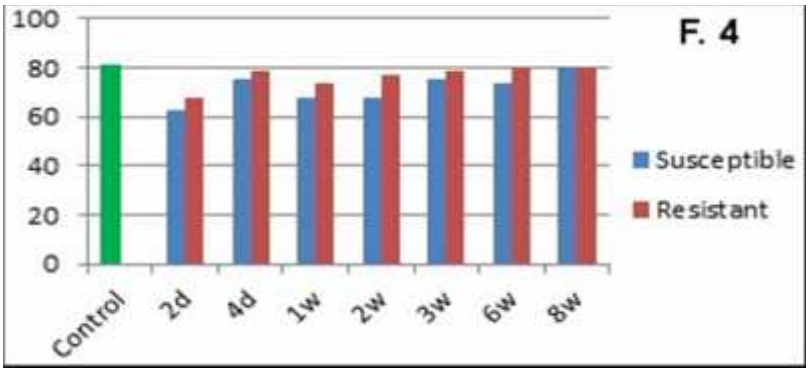
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