Original Article

Morphology and kinetics of susceptible and resistant *Biomphalaria alexandrina* hemocytes during the first week of exposure to *Schistosoma mansoni* miracidia

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ABSTRACT

Background: Few studies concerning *Biomphalaria alexandrina* (*B. alexandrina*) snail hemocytes' subpopulations, and their relation to the compatibility with *Schistosoma mansoni* (*S. mansoni*)' miracidia were performed. Manipulation of parasite development inside these snails could be applied as a control measure against schistosomiasis.
Objectives: Knowing that the snail hemocytes temporarily bind to the parasites, allowing the development of cercariae that are infective to the definitive host. This study aimed at studying the morphology and kinetics of diverse hemocytes of susceptible and resistant *B. alexandrina* and their participation in the snail early immune response after challenge by *S. mansoni*.
Material and Methods: Giemsa stained hemocytes were characterized using light microscopy. Total and differential hemocyte counts (THC and DHC) were calculated in the hemolymph of two groups composed of 60 susceptible and 60 resistant snails. Each group was further subdivided as 12 control pre-exposure snails (PE) and 48 post-exposure snails (PO) to *S. mansoni* at different time points (6 h, 1, 3 and 7 days). THC and DHC counts were recorded by a snail hemogram.
Results: Results revealed that granulocytes constituted the most common population all through the experiment with the large dense-granulated granulocytes subpopulation being the largest-sized cells detected. The highly reactive subpopulations that increased in number upon exposure to *S. mansoni* were the few-granulated and the large-granulated granulocytes, suggesting their possible participation in early parasite destruction.
Conclusion: The resulting hemograms helped determine the participation of hemocyte populations and subpopulations in the defense against *S. mansoni*, aiding in understanding snail compatibility patterns. Further studies to propagate transgenic *B. alexandrina* snails abundant in large granular granulocytes utilizing (gene editing) CRISPR-Cas9 technique are recommended. This would be required to spread schistosome resistance traits in snail populations, thus, contributing to reduced schistosomiasis transmission in the long run.

Keywords: *Biomphalaria alexandrina*, blast-like cells, differential hemocyte count, granulocytes, hyalinocytes, total hemocyte count.

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INTRODUCTION

B. alexandrina snail acts as an obligate intermediate host for *S. mansoni* miracidia, a parasite that causes intestinal schistosomiasis, a major neglected tropical disease, that affects millions all over the world[1]. Different *Biomphalaria* species transmitting *S. mansoni* exist in Africa and South America. Among the African species, *B. alexandrina* snail acts as the main intermediate host in Egypt[2].

For completion of *S. mansoni* life cycle, mature eggs are excreted in the definitive host feces. On reaching freshwater, the eggs hatch releasing freely swimming miracidia which on penetration of the snail, stimulate the snail defense system. At the same time the parasite tries to escape the snail host weapons in order to propagate its life cycle[3,4]. Compatible snails allow successful schistosome development, while incompatible snails' internal defense system destroys the invading parasite[5]. The *Biomphalaria* internal defense system consists of humoral and cellular elements. Hemocytes populate the snail circulatory system and constitute the principal cellular defense line[6,7]. Hemocytes of resistant snails destroy parasite larvae by phagocytosing their tegument and producing reactive oxygen intermediates. Activation of the internal defense also occurs during parasite development inside susceptible snails, where, hemocytes temporarily bind to the parasites, allowing the development of sporocysts then cercariae. The latter are shed in freshwater to infect the definitive host[5,8,9].

Despite the considerable efforts spent on characterizing hemocytes in different mollusks, there are many controversies concerning their classification[10]. Hemocytes of various *Biomphalaria* species have variable morphology and different

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subpopulations[11-13]. Nevertheless, several authors agreed that *Biomphalaria* hemocytes consist of two primary cell populations: granulocytes that are mainly involved in phagocytosis and cytotoxicity with generation of reactive ions from their cytoplasmic granules, and hyalinocytes that are less phagocytic due to absence of cytoplasmic granules[14-16]. A third hemocyte population, the blast-like cells, has been characterized. Being small cells with a large nucleus, they are thought to be the progenitors of the other populations[11,17]. Regarding *B. alexandrina* snails, previous research studied their hemocytes[18-21], yet, information about the functional attributes of their populations and subpopulations in response to *S. mansoni* infection is still deficient.

Identification of *B. alexandrina* hemocyte dynamics is essential in understanding compatibility differences among various *Biomphalaria* species, which in turn can help in providing new approaches to break the *Schistosoma* transmission cycle inside its intermediate host[8,22]. Therefore, in the current work, the morphology of Giemsa stained *B. alexandrina* hemocytes and their participation in the early immune response of susceptible and resistant snails against *S. mansoni* miracidia were studied using light microscopy.

MATERIAL AND METHODS

This case-control study was conducted at the Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt during the period from May to August, 2016.

Snails and parasite: *B. alexandrina* snails were originally obtained from water channels in Alexandria (Egypt) during the year 2011. Since then, snails were maintained under suitable environmental conditions; breeding in aquaria containing dechlorinated tap water at 26 °C, and were fed lettuce leaves supplemented with fish food and calcium carbonate[4].

S. mansoni strain sympatric to the bred *B. alexandrina* strain was used in this study[23]. The parasite was originally obtained by shedding cercariae from naturally infected *B. alexandrina* snails. Maintenance of *S. mansoni* life cycle was conducted between the laboratory-bred snails and male Swiss-albino mice. Using the paddling technique, each mouse was infected with 120 cercariae[24]. Seven to eight weeks post-infection, *S. mansoni* eggs obtained from mice, were exposed to direct sunlight to stimulate miracidial hatching. Two-months-old snails were challenged by individual exposure to 10 active miracidia[8].

For separation of susceptible and resistant snail stocks, snails that showed high infection rates were isolated and reared singly for self-reproduction and their progeny was identified as the susceptible stock. While snails that remained uninfected after two exposures were isolated and reared singly for self-reproduction. Their progeny was the resistant stock[9]. From the two progenies, 60 susceptible and 60 resistant snails, sized about 8 mm, were selected to comprise the susceptible and the resistant groups, respectively. In each of the two groups; twelve snails remained unexposed to infection (2 control subgroups). The remaining 48 snails in each of both groups were exposed individually to ten *S. mansoni* miracidia (2 exposed subgroups).

Hemolymph collection: Snails shells were wiped with 70% alcohol and dried with an absorbent paper. Hemolymph collected by cardiac puncture was placed in a siliconized tube in an ice bath to prevent cellular aggregation and to permit the settling and removal of any debris[9]. Hemolymph collection was performed at 6 h, 1d, 3d, and 7d post individual exposure (PO) to 10 *S. mansoni* miracidia. These were compared to the control unexposed subgroups that were considered as the base line hemocyte counts (0 hour). The experimental design is described in figure (1).

Total hemocyte counts (THC): The number of hemocytes in 1 µl of fresh hemolymph from each of the 12 individual snails (biological replicates) was counted using a Neubauer chamber under bright-field microscopy at the high-power magnification (X40). The count was repeated 6 times for each sample at each time point (technical replicates)[11].

Hemocyte characterization: One µl of the hemolymph from each snail was allowed to dry on a microscope slide for 30 min, then, fixed in methanol for 10 min, stained with Giemsa (diluted 1:9 in buffered distilled water) for 10–15 min. The slides were then washed with buffered distilled water and dehydrated in ethanol, then examined using bright-field microscopy at X100 magnification for morphological identification of the cells. To determine the nucleus/cytoplasm (N/C) ratio, the cellular and nuclear dimensions of 50 cells from each subpopulation were measured using an eyepiece micrometer. The measurements (µm) were taken at the broadest diameter, excluding the pseudopodia and filopodia[11,25].

Differential hemocyte counts (DHC): To determine the mean value of each hemocyte population and subpopulation, the numbers of morphologically different hemocytes were counted in Giemsa stained smears, from the same samples that were used for THC. Based on counting ~ 200 cells/snail, the mean value of each hemocyte type was calculated. The same counting methodology was repeated 6 times (technical replicates) for each snail.

Statistical analysis: Quantitative data were analyzed using *F*-test (ANOVA) and post-hoc tests. Data are expressed as means ± standard deviation (SD). $P<0.05$ was considered statistically significant. Statistical

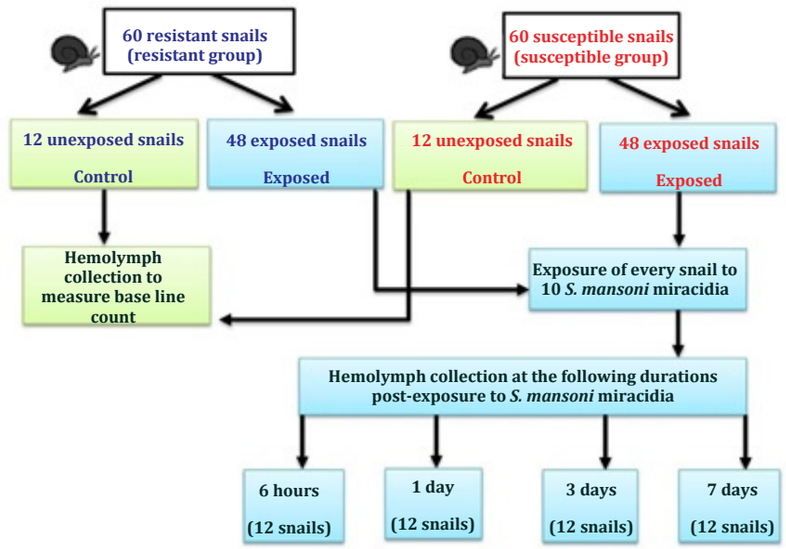

Fig. 1. Experimental design.

calculations were performed using IBM SPSS software package version 20.0 (SPSS Inc, Chicago, IL, USA).

Ethical approval: All experiments were conducted abiding with institutional ethical guidelines for animal use in research. The study protocol was approved by the Ethics Committee of Faculty of Medicine, Alexandria University, Egypt.

RESULTS

Total hemocyte counts (THC): The mean THC of susceptible unexposed control snails was 406.17±12.51 cells/µl, which was significantly lower than the mean THC of the resistant unexposed controls (424.83±6.97 cells/µl). After exposure to *S. mansoni*, THC showed remarkable changes in susceptible and resistant groups, at different experimental time periods. In both groups, the THC was significantly lower than that of the corresponding control group at 6 h PO. This was followed by an increase at the 1st day post exposure (DPO) and maintained at a high level till the end of the experiment. Full data concerning THC are shown in figure (2). Table (1) displays the intergroup comparison between THC and DHC of susceptible and resistant groups at different time periods PO to *S. mansoni* miracidia.

Hemocyte characterization (morphology): Based on nucleus/cytoplasm sizes, the N/C ratio, the presence of cytoplasmic granules and cytoplasmic extensions, three main hemocyte populations were identified in *B. alexandrina* hemolymph. These were: blast-like cells, hyalinocytes, and granulocytes[11]. Table (2) shows the hemocytes measurements.

Population I: Blast-like cells (BL) were the smallest cells with a mean diameter of 4.0±0.2 µm. They were round, lymphocyte like, having a relatively large, dark-stained basophilic central or eccentric nucleus, which occupied almost the entire cell with a thin rim of blue cytoplasm. Hence, they had the highest N/C ratio of about 0.8±0.03, which was significantly different from other populations. The cytoplasm did not show any extensions (Fig. 3). Two subpopulations were identified: agranular blast-like cells with no granules (Ag BL) or subpopulation Ia, and granular blast-like cells (GBL) or subpopulation IIa that contained basophilic granules.

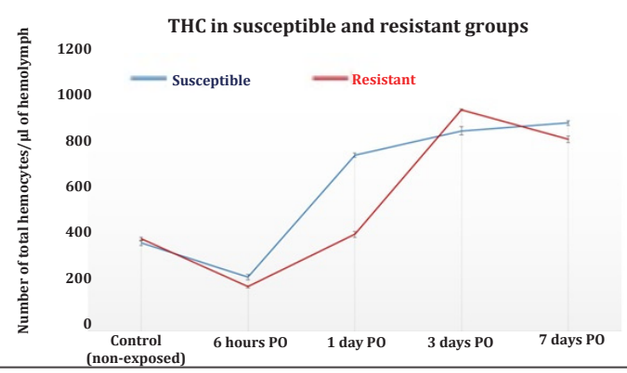

Fig. 2. Intergroup comparison between the total hemocyte count (THC) of susceptible and resistant groups before and after exposure to *S. mansoni* miracidia. **PO:** Post-exposure to *S. mansoni*.

Table 1. Intergroup comparison between the means of THC and DHC of susceptible and resistant groups at different experimental periods.

Hemocyte Pop & SubPops		Time of exposure									
		Control (0 hour)		6 hours PO		1 day PO		3 days PO		7 days PO	
		S	R	S	R	S	R	S	R	S	R
THC		406.17±12.51	424.83±6.97	250±11.82	205.83±4.84	810.67±8.91	446.83±13.1	923.33±18.51	1020.67±13.6	959.17±9.87	884±14.04
		P*=0.02		***P*=0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***	
Pop I (B)	TBL	39.83±1.17	42.67±3.27	29±5.33	37.67±2.88	99±4.29	83.83±2.71	131.17±5.46	128±4.03	133±2	113.33±4.37
		P* = 0.08		***P* = 0.02***		***P* = 0.00***		***P* = 0.05***		***P* = 0.00***	
	AgBL	33.17±2.56	36.17±3.19	22±3.79	28.67±2.73	72.17±4.26	60±2.61	108.67±6.74	102±1.9	107±3.16	96±2.97
		P* = 0.09		***P* = 0.02***		***P* = 0.00***		***P* = 0.01***		***P* = 0.00***	
	GBL	33.17±2.56	36.17±3.19	22±3.79	28.67±2.73	72.17±4.26	60±2.61	108.67±6.74	102±1.9	107±3.16	96±2.97
		P* = 0.79		***P* = 0.02***		***P* = 0.01***		***P* = 0.24**		***P* = 0.00***	
Pop II (H)	TH	129±6.16	119.5±1.76	52.17±5.19	75.67±4.63	134.67±3.78	161.5±3.73	218.83±10.57	100.17±1.6	242.5±7.03	112±2.68
		P* = 0.00		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***	
	SH	61.17±2.71	55.17±3.19	29.67±3.2	38.83±2.31	65.17±2.79	76.83±2.48	130±5.76	48.17±1.94	133±2.97	56.5±2.74
		P* = 0.01		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***	
	LH	67.83±4.31	64.67±3.14	22.5±2.17	36.83±2.32	69.5±2.35	84.67±3.27	88.83±6.43	52±2.1	109.5±5.36	55.5±3.73
		***P* = 0.17**		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***	
Pop III (G)	LDgG	237.33±6.31	262.83±5.42	168.83±4.45	92.5±1.38	577±6.81	205±3.52	573.33±7.45	792.67±3.67	583.67±3.98	640.83±31.52
		***P* = 0.17**		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.06***	
	Sg FgG	103.17±3.31	115.33±2.8	65.67±5.28	36.33±2.94	228±7.24	91.17±1.94	250.5±8.48	344.67±3.78	244.33±2.88	282.17±5.19
		P* = 0.00		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***	
	Sg SDgG	44.17±2.13	46.83±2.56	27.5±2.74	22.67±3.67	97.67±4.27	49.33±2.73	88.67±6.15	133.17±2.56	105.67±2.8	111.5±4.72
		P* = 0.00		***P* = 0.03***		***P* = 0.00***		***P* = 0.00***		***P* = 0.02***	
	LDgG	87.67±4.8	119±39.25	77.33±3.01	34±2	251.33±2.25	65±1.79	234.17±4.88	314.83±2.32	233.67±3.88	263.83±4.17
		P* = 0.00		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***		***P* = 0.00***	

***:** Significant at ≤0.05. **Ag BL:** Agranular blast-like cells; **FgG:** Few-granulated granulocytes; **GBL:** Granular blast-like cells; **LH:** Large hyalinocytes; **LDgG:** Large dense-granulated granulocytes; **PO:** Post-exposure to *S. mansoni*; **Pop:** Population; **Pop I (B):** Blast-like cells; **Pop II (H):** Hyalinocytes; **Pop III (G):** Granulocytes; **R:** Resistant; **S:** Susceptible; **SDgG:** Small dense-granulated granulocytes; **Sg:** Small granulocytes; **SH:** Small hyalinocytes; **SubPops:** Subpopulations; **TBL:** Total blast-like cells; **TH:** Total hyalinocytes; **THC:** Total hemocyte count.

Table 2. Measurements of Giemsa stained *B. alexandrina* hemocytes.

Hemocyte sub-population			Cell dimension (µm)	Nucleus dimension (µm)	N/C ratio
Pop I (B)		AgBL (Ia)	4.0 ± 0.2	3.2 ± 0.1	0.8 ± 0.03
		GBL (Ib)	4.0 ± 0.2	3.2 ± 0.1	0.8 ± 0.3
Pop II (H)		SH (IIa)	4.5 ± 0.02	2.8 ± 0.1	0.6 ± 0.02
		LH (IIb)	6.4 ± 0.2	2.3 ± 0.1	0.4 ± 0.02
Pop III (G)	SG (IIIa)	FgG with lobulated nucleus	4.4 ± 0.4	2.4 ± 0.1	0.5 ± 0.3
		SDgG with blue round nucleus	5.5 ± 0.2	1.0 ± 0.2	0.2 ± 0.3
	LDgG (IIIb)		13.5 ± 0.5	3.5 ± 0.1	0.3 ± 0.1

AgBL: Agranular blast-like cells; **FgG:** Few-granulated granulocytes; **GBL:** Granular blast-like cells; **H:** Hyalinocytes; **LDgG:** Large dense-granulated granulocytes; **LH:** Large hyalinocytes; **Pop:** Population; **N/C:** Nucleocytoplasmic ratio; **SDgG:** Small dense-granulated granulocytes; **SG:** Small granulocytes; **SH:** Small hyalinocytes. There is a statistically significant difference between the different cell populations and subpopulations with regard to N/C ratio (F = 5947.417).

Population II: Hyalinocytes (H) were round to oval cells, characterized by their homogenous pale blue cytoplasm, and a basophilic eccentric nucleus (paler and smaller than in blast-like cells). The cytoplasm was full of variably sized vacuoles with no granules nor cytoplasmic extensions (Fig. 4). Hyalinocytes included two subpopulations according to the size of the cell and the nucleus: small hyalinocytes (SH) or subpopulation IIa with a mean diameter of 4.5±0.02 µm and N/C ratio of 0.6±0.02 and large hyalinocytes (LH) or subpopulation IIb with a mean diameter of 6.4±0.02 µm and N/C ratio of 0.4±0.02.

Population III: All granulocytes (G) exhibited cytoplasmic granules that sometimes pushed the nucleus to the periphery. Some had the capability of forming prominent pseudopodia or filopodia. They were variable in size and polymorphic, from circular to fusiform. Some showed two nuclei and a dividing cytoplasm representing cell division. They were

differentiated into:

(a) Small granulocytes (subpopulation IIIa): Those were subdivided according to the staining characteristics and shape of the nucleus into:

- **Subpopulation IIIa1 (few small-granulated granulocytes):** These were polymorphic; round, oblong to spindle. They were small with a mean diameter of 4.4±0.4µm, and showed small lobulated basophilic nucleus, few basophilic peripheral granules, prominent vacuoles, short and long filopodia and tended to attach (Fig. 5).
- **Subpopulation IIIa2 (small dense-granulated granulocytes):** These were round cells with a mean diameter of 5.5±0.2µm. They had small round blue nuclei that were centrally located or peripherally displaced. Basophilic granules filled their cytoplasm (Fig. 6).

(b) Large granulocytes (subpopulation IIIb): These were round large cells with a mean diameter of 13.5±0.5µm. They had relatively small blue nuclei, hence, a low N/C ratio of about 0.3±0.1. Nuclei were located centrally or were peripherally displaced. Cytoplasmic granules were either basophilic or acidophilic. Granules were either fine (Fig. 7a) or occasionally coarse, and deeply stained (Fig. 7b). Some showed thick pseudopodia (Fig. 7c). Rarely, large cells with the same staining characteristics of large granulocytes were seen. However, they had pinkish cytoplasm with no granules, but instead contained many small vacuoles. These cells were identified as

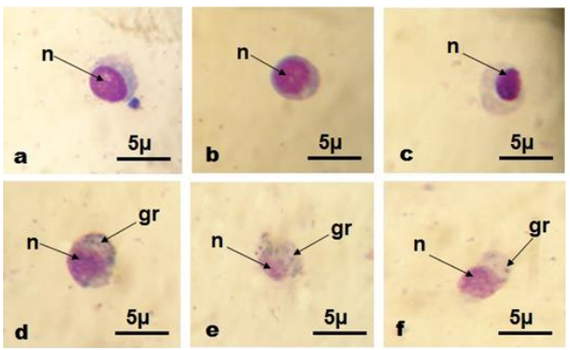

Fig. 3. Light micrographs of Giemsa stained blast-like cells of *B. alexandrina* snails: **a-c:** Agranular blast-like cells with an eccentric large nucleus **(n)** surrounded by a thin rim of basophilic cytoplasm without granules; **d-f**: Granular blast-like cells with an eccentric large nucleus **(n)**, with basophilic cytoplasm containing granules **(gr)**.

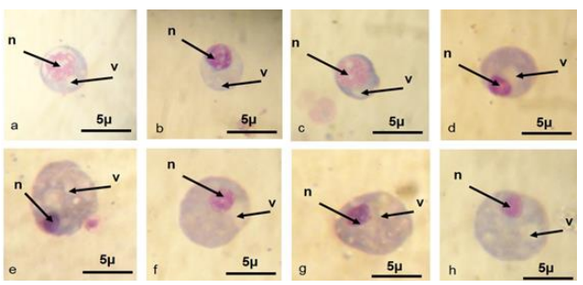

Fig. 4. Light micrographs of Giemsa stained hyalinocytes of *B. alexandrina* snails. **a- d:** Small hyalinocytes with a basophilic nucleus **(n)** and pale blue cytoplasm without granules nor cytoplasmic extensions, but with vacuoles **(v)**; **e-h:** Large hyalinocytes with a small peripheral nucleus **(n)**, pale blue cytoplasm and vacuoles **(v)**.

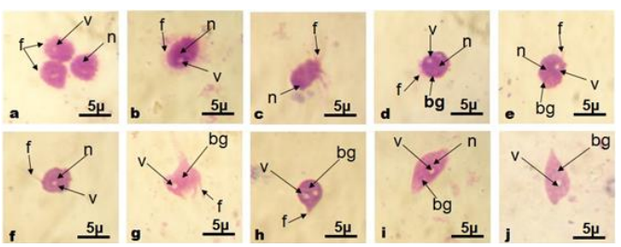

Fig. 5. Light micrographs of Giemsa stained hyalinocytes of *B. alexandrina* snails. **a- d:** Small hyalinocytes with a basophilic nucleus **(n)** and pale blue cytoplasm without granules nor cytoplasmic extensions, but with vacuoles **(v)**; **e-h:** Large hyalinocytes with a small peripheral nucleus **(n)**, pale blue cytoplasm and vacuoles **(v)**.

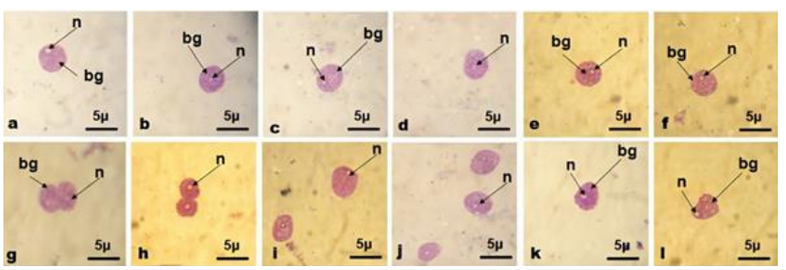

Fig. 6. Light micrographs of Giemsa stained small dense-granulated granulocytes of *B. alexandrina* snails, with round blue stained nucleus and basophilic granules **(bg)**: **a-f**: regular small granulocytes; **g-h:** dividing forms; **i:** irregular small granulocytes.

'vacuolated cells' (Fig. 7d). They constituted less than 0.5 % of the THC.

Differential hemocyte counts (DHC): Means of different populations and subpopulations in the susceptible and resistant groups are shown in table (1). Blast-like cells (population I) and their subpopulations showed the least mean among the hemocytes in all studied groups. Conversely, granulocytes (population III) were found to be the most common cell type in the hemolymph of the susceptible (58.4±.4.1%) and resistant (61.9±0.75%) control snails. Intragroup comparisons for DHC are shown in figure (8) for populations, and in figure (9) for subpopulations.

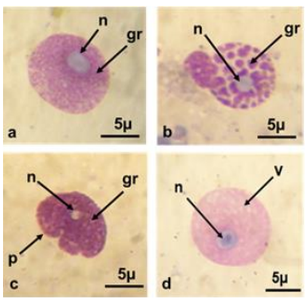

Fig. 7. Light micrographs of Giemsa stained *B. alexandrina* large granulocytes. **a:** with fine basophilic granules **(gr)**, nucleus **(n)**; **b:** with coarse granules **(gr)**, nucleus **(n)**; **c:** with well-developed pseudopodia **(p)**; **d:** vacuolated cell with blue-stained nucleus **(n)** and many vacuoles **(v)**.

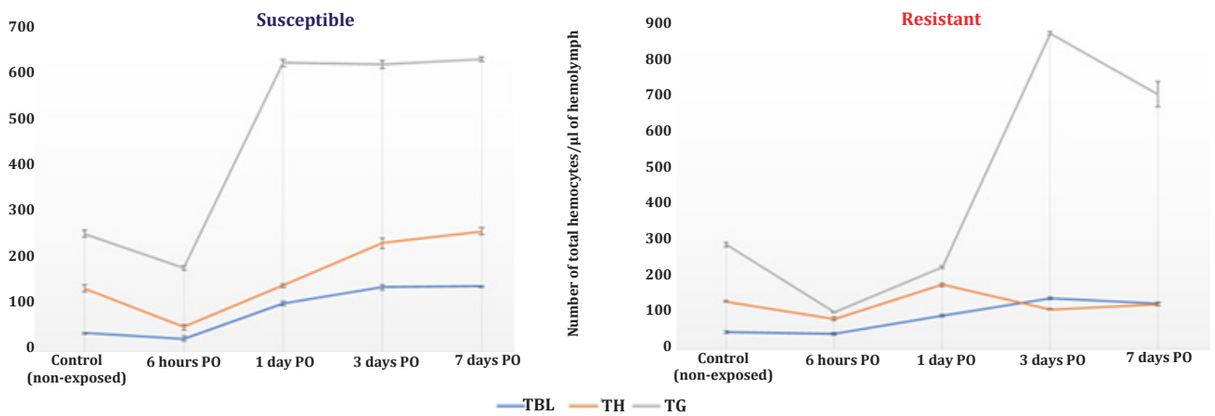

Fig. 8. Comparison of differential hemocyte count (DHC) among susceptible and resistant populations. **PO:** Post-exposure to *S. mansoni*; **TBL:** Total blast-like cells; **TG:** Total granulocytes; **TH:** Total hyalinocytes.

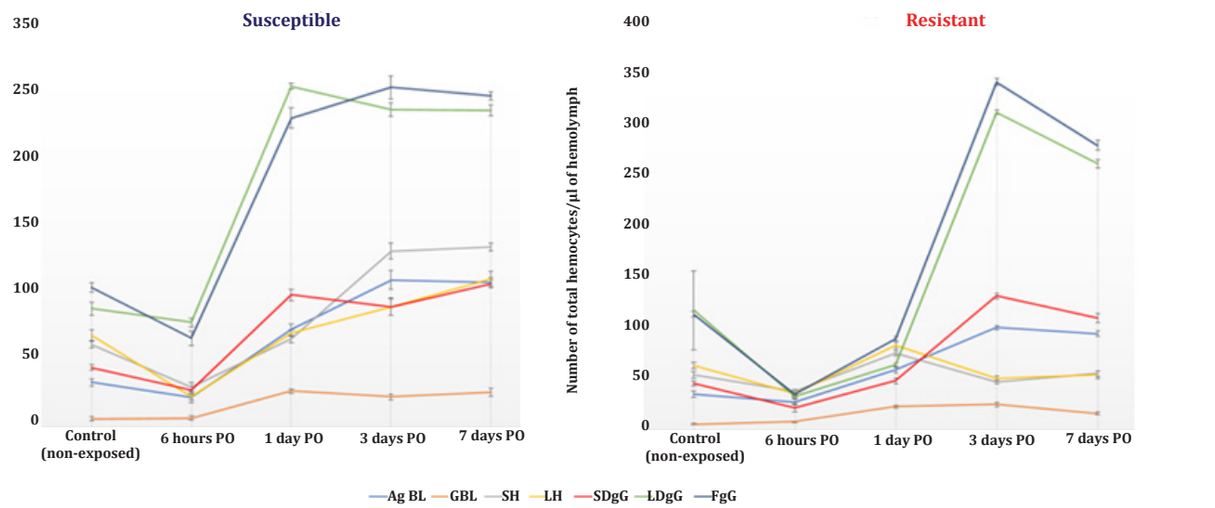

Fig. 9. Comparison of differential hemocyte count (DHC) among susceptible and resistant subpopulations. **Ag BL:** Agranular blast-like cells; **FgG:** Few-granulated granulocytes; **GBL:** Granular blast-like cells; **LDgG:** Large dense-granulated granulocytes; **LH:** Large hyalinocytes; **PO:** Post-exposure to *S. mansoni*; **SDgG:** Small dense-granulated granulocytes; **SH:** Small hyalinocytes.

DISCUSSION

The present study confirmed that because of their state of resistance, the THC in the unexposed resistant *B. alexandrina* snails was significantly higher than in the unexposed susceptible snails. This finding followed the report by Walker and Rollinson[26], yet contradicted that of Abaza *et al*.[13].

The recorded current THC complies with those obtained by Bakry[19] and Osman *et al*.[27], but are far from the THC obtained by Mohamed[20]. Variation in the hemocyte count among different species and even within the same species was previously mentioned[22,28,29]. Besides, significant THC differences between the exposed (susceptible and resistant) snails and their controls were observed, at different experimental periods. The only THC reduction in both groups below their controls was achieved at 6 h PO. This could be explained by hemocyte migration towards the recently attacked tissue. This decrease was noted more in resistant snails. Lockyer *et al*.[30] attributed the great migratory capacity of resistant hemocytes to the presence of cell surface receptors that bind to the extracellular matrix, thus, enhancing cell migration through the tissues towards the invading pathogens. The investigators found that resistant hemocytes express genes that are related to the actin function, therefore, increasing cellular movements, which are either concerned with the interaction of granulocytes with the parasite or with repair of injured tissues by hyalinocytes following the parasitic invasion[30]. A similar early reduction in THC was previously obtained in *B. alexandrina*[20], *B. glabrata*[31], and *B. tenagophila*[28,31]. Moreover, an early migration occurred during *in vitro* interaction between *S. mansoni* sporocysts and *Biomphalaria* hemocytes[5,9].

Following this early THC reduction, a significant increase at the 1st DPO in both groups was detected especially in the susceptible group. This finding was in agreement with de Melo *et al*.[32], who stated that the response at the 1st DPO was more potent than the response during earlier stages of host-parasite interaction. According to Araque *et al*.[33], the early migration of hemocytes to the internal tissues happens regardless of snail-parasite compatibility status. After this stage, the process of parasite infection differs between susceptible and resistant snails, where, resistant snails recognize the parasites as non-self, thus forming a strong connection between snails' hemocytes and the parasite, leading to persistence of the hemocytes in resistant snails' tissues for a longer time than in case of susceptible snails[28]. In susceptible snails, hemocytes are activated by the invading parasite, yet, they cannot encapsulate the invader due to absence of lectins that firmly connect the parasite and the hemocytes, thus, they returned to the hemolymph at this time[31].

The increased THC in both groups at the 1st, 3rd, and 7th DPO could be attributed to the production of new hemocytes from the amebocytes producing organ (APO) and other snail hemopoietic centers[16]. According to two reports[34,35], *S. mansoni* extracts maximally stimulate hemocyte production from APO within 48 hours and not earlier. This explains why the current maximum THC elevation was achieved at the 3rd day PO in the resistant group. Similarly, a significant THC increase was recorded at the 3rd and 7th DPO[27], while others recorded a significant THC reduction at the 7th DPO[20,29].

Different theories explained hemocyte differentiation. One of them proposes that there is only one blast cell line that gives rise to hyalinocytes, which might later become granulocytes[15,17]. Another assumption is the existence of more than one lineage that gives rise to different hemocytes[36,37]. The term 'cell population' was implemented herein, instead of 'cell type' because the functional identification of the cell types was not performed[38].

The blast-like cells were the least encountered all through the current experiment in both groups. They showed the same morphology as those belonging to *B. glabrata* and *B. straminea*[11], gastropods[17,37], bivalves[39], and other invertebrates[25]. Hine[15] assumed them to be hemocyte progenitors. In the present work, the recorded unique granular blast-like cells may be the progenitor of future granulocytes.

The hyalinocytes described in this work had the same morphology of hyalinocytes in different mollusks[15,40,41]. Barracco *et al*.[41] and Cheng and Auld[42] noted that the hyalinocytes of *B. glabrata* and *B. tenagophila*, respectively, were poorly spreading cells in the contrary to granulocytes that readily spread on surfaces. On the other hand, Cavalcanti *et al*.[11] found many hyalinocytes with filopodia in *B. glabrata* and *B. stramenia*.

Interestingly, small hyalinocytes currently detected were found to have a higher N/C ratio, than large hyalinocytes. According to Donaghy *et al*.[17], the increase in cell size and the decrease in the N/C ratio from small to large indicates cell maturity. Souza Sdos and Andrade[16] described hyalinocytes as immature, small spherical cells, versus granulocytes that exhibited eccentric nuclei and expanded cytoplasm with filopodia.

Granulocytes shown in the present study were detected more in resistant snails[13]. They possessed well-developed pseudopodia or filopodia resulting in cell-to-cell clumping[37]. They contained many granules similar to those of *B. glabrata*[14,42], except for granulocytes in subgroup IIIa1 that showed fewer

granules in agreement with Helal *et al*.[18]. Barracco *et al*.[41] showed that *B. tenagophila* granulocytes contained few granules. It is noteworthy that granulocytes with two nuclei and a dividing cytoplasm were commonly detected in resistant snails suggesting cell multiplication[13].

Based on their affinity to Giemsa stain, the large granulocytes detected herein contained basophilic or eosinophilic granules, in agreement with Bakry[19]. On the other hand, Cavalcanti *et al*.[11] found that granules in *B. glabrata* and *B. stramenia* granulocytes were mostly basophilic. Cheng[14] stated that granules' different staining properties may be explained by pH changes and ontogenetic an/or metabolic phases within the granulocyte line.

Granulocytes containing large coarse granules were occasionally detected. These cells are known to transport metabolic substances from the digestive glands, or maybe involved in the aggregation process[40,43].

Vacuolated cells were scarcely detected (less than 0.5%). They shared morphological similarities with granulocytes, yet lacked granules and possessed many vacuoles. These cells could not be classified before[39]. With regards to DHC, blast-like cells showed variations in their count along the whole experiment, possibly because they act as stem cells for other cell populations[44].

Granulocytes are the main cells engaged in snail defense[6,9], thus, variations in their count upon facing parasitic infection are more likely to occur than variations in hyalinocytes' count[28,45]. This explains our results which showed that granulocytes were significantly higher in the control resistant snails than the corresponding susceptible snails, while hyalinocytes displayed a reverse profile. Allegretti *et al*.[46] attributed the higher resistance of albino *B. glabrata* snails to *S. mansoni* infection to their increased granulocytes count. In the same context, Pila *et al*.[47] suggested that high granulocytes' count is related to resistance. Working on *B. glabrata*, Cheng and Auld[42] reported that granulocytes comprised 93% of THC. Similarly, Barracco *et al*.[41] stated that *B. tenagophila* granulocytes represented 90% of the circulating hemocytes. Conversely, Cavalcanti *et al*.[11] reported that granulocytes constituted only 4% of *B. glabrata* THC and 5% of *B. stramenia* THC.

In our study following exposure to *S. mansoni*, hyalinocytes' count decreased within 6 h in both susceptible and resistant snails. This may be due to their role in tissue repair and coagulation and in their immune response[48]. Osman *et al*.[27] found that hyalinocytes were capable of phagocytosis but, at a less extent than granulocytes.

The granulocyte count decreased 6 h PO, especially in resistant snails. This decrease was followed by a significant elevation from the 1st DPO in susceptible snails and the 3rd DPO in resistant snails and continued to the 7th DPO in both snail groups. The delay in count elevation in resistant snails could be due to activation of the granulocytes by the parasite excretory-secretory products, with subsequent parasitic migration and adhesion[5,49]. The elevation of granulocyte count thereafter could be due to the stimulation of hemopoiesis.

The exposure to *S. mansoni* led to great variations in the number of the granulocytes subpopulations, reflecting differential involvement in the subpopulation response to the parasite. Total granulocytes count has increased at the 3rd and the 7th DPO in resistant snails. The large dense-granulated granulocytes showed the same increase in number highlighting their cytotoxic defense role against the parasite. Degranulation of the granulocytes leads to release of reactive components causing parasite lysis and death[50].

Few-granulated granulocytes showed the same kinetic changes in their number, suggesting that they have the same defense function. This granulocyte subset, in particular, was very reactive upon *S. mansoni* exposure indicating its participation during early parasite destruction. Consequently, granulocytes' subpopulations that were highly reactive against *S. mansoni* in the present study were the few-granulated granulocytes and the large-granulated granulocytes. Martins-Souza *et al*.[22] proved that these subpopulations are responsible for *S. mansoni* destruction in *B. tenagophila*. Meanwhile, the number of the small dense-granular granulocytes' subpopulation did not show significant changes, compared to the total granulocytes, all through the experiment in susceptible and resistant snails. This could reflect the failure of these cells to participate in immune defense against *S. mansoni* infection. Yoshino *et al*.[51] demonstrated that small, rounded granulocyte subpopulation in *B. glabrata* lacks certain glycotopes, which are important for parasite killing.

Interventions targeting the snail hosts are crucial in the integrated strategy required for schistosomiasis elimination and include the recent revolution in gene drive technology, gene editing or clustered, regularly interspaced short palindromic repeats (CRISPR) tools. CRISPR-Cas9 was recently used to identify and control vector tick embryos' genes, rendering them resistant to Lyme disease, thus decreasing disease transmission[52,53]. This technique could help spread schistosome resistance traits in snails' population, thus, reducing schistosomiasis transmission in the long run[52].

In conclusion, the current study reveals differences in the kinetic response of hemocytes between susceptible and resistant snails during the first week after exposure to *S. mansoni*. The differential responses at this critical period are related to the host-parasite interactions with subsequent effects on the parasite propagation inside the snail. Hemograms obtained could help in determining the participation of each cell population and subpopulation in the defense response against *S. mansoni* infection. This would serve as useful background data for future studies concerning *Biomphalaria* hemopoiesis and ontogenesis. Better understanding of the snail compatibility patterns allows the developing of new modalities that enhance hemocyte activity that increases resistance to infection. Further studies to utilize gene editing techniques and CRISPR-Cas9 technique for propagation of transgenic strains of *B. alexandrina* snails are required to spread schistosome resistance traits in snails' population, thus, contributing to reduced schistosomiasis transmission in the long run.

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