Immune responses of the circulating haemocytes of *Lissachatina fulica* (Bowdich, 1822) challenged with the spores of *Rhizopus* sp and *Aspergillus* sp.

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ARTICLE INFO
Received: 20.06.2021
Revised: 07.08.2021
Accepted: 16.08.2021

Key words:
Fungal spores, Gastropoda, Phagocytosis, Pleomorphic fibrillar cell, SEM, Time lag immune response.

ABSTRACT

Light and scanning electron microscopic observations are made on the haemocytes in the haemolymph of *Lissachatina fulica*. Three distinct populations of the haemocytes are recognized. They are Group I, Group II, and Group III haemocytes. Group I cells are round, non-spreading small cells. Group II and Group III cells are spreading haemocytes. Group II cells are categorized into agranulocytes, small granulocytes, and large granulocytes. Agranulocytes are large slightly spreading agranular cells. Small granulocytes are small spreading granular cells. Large granulocytes are spreading granular cells, forming numerous pseudopodia. Group III cells are termed as "pleomorphic fibrillar cells". Group III cells are characterised into three sub-types (Type-A, Type-B, and Type-C) depending on cell morphology. Time lag immune responses of all the haemocytes are studied after challenged with the spores of *Rhizopus* sp and *Aspergillus* sp. Group I cells show their defense response by formation of the cell nodulation to each other. Cell nodulation is very common response of the agranulocytes, small granulocytes, Type-A and Type-B Group III cells against the invading fungal spores. Large granulocytes form numerous pseudopodia and vacuoles. It is observed that the circulating haemocytes in the snail showing more cell surface modifications after challenged with the spores of *Rhizopus* than that of the spore of *Aspergillus*. The study advocates that the immune responses of the circulating haemocytes of *Lissachatina fulica* may depends on the specific immune activator.

Foot notes: The co-authors Dr. Korak Kanti Chaki and Dr. Kamales Kumar Misra were unfortunately expired on June 5th 2021 and Feb 23rd 2021 respectively. This work could not complete without their encouragement and cooperation. We are feeling very sad about their untimely departure and this work is dedicated to their unforgettable memories.

Introduction

Invertebrate immune systems apparently lack immunoglobulins, interactive lymphocyte subpopulations and lymphoid organs but they depend on circulating haemocytes (or immunocytes). Nevertheless, the huge numbers and diversity of invertebrates attests to the efficiency of their host defense. The mucus and tough external skeletons form barriers to invasion in some coelenterates and molluscs, echinoderms, and arthropods. When these barriers are breached, the pathogens are then exposed to a range of interacting cellular and humoral defense reactions like, blood clotting/coagulation, wound healing, phagocytosis, encapsulation responses, natural and inducible antimicrobial factors. Circulating immunocytes are considered as main responsive cells in the immune system of gastropods. Haemolymph of gastropod molluscs contains dissolved haemoproteins and immunocytes which mainly perform phagocytosis (Sminia, 1972, 1981; Yoshino, Granath, 1985; Knaap et al., 1981). Phagocytic cells ingest microbial as well as other immunogenic invaders and encircle larger invaders. Light and electron microscope studies revealed that there are two categories of immunocytes in gastropod molluscs as either agranulocytes or hyalinocytes and granulocytes (Cheng, 1984; Yonow, Renwrantz, 1986; Voltzow, 1994; Mahilini & Rajendran, 2008) or non-spreading, round cells and spreading cells (Ottaviani, 1983; Ottaviani, 1992; Ottaviani & Franchini, 1988; Martin et al., 2007).

The structure and functions of haemocytes are studied in a number of gastropods including pulmonates such as *Lymnaea stagnalis* (Adema et al., 1992; Adema et al., 1994), *Biomphalaria glabrata* (Matricon-Gondran, Letocart, 1999), *Haliotis tuberculata* (Serpentini *et al.*, 2000; Malham et al., 2003), *Megathura crenulata* and *Aplysia californica* (Martin et al., 2007), *Trachea vittata* (Mahilini & Rajendran, 2008) and *Pila globosa* (Mahilini & Rajendran, 2008; Ray et al., 2013), *Bellamya bengalensis* (Kambale & Potdar, 2010; Ray et al., 2013). The types and functions of haemocytes in the molluscs in ambient conditions are necessary in studying basic cell responses to the infections (Beckmann et al., 1992; Medzhitov, Janeway, 2000; Janeway & Medzhitov, 2002; Cochennec-Laureau et al., 2003; Plows et al., 2005; Ottaviani, 2011).

The giant African snail, *Lissachatina fulica* (Bowdich, 1822) (Achatinidae, Stylommatophora, Eupulmonata) is considered as an agricultural pest throughout the World. This species is formerly known as *Achatina fulica* (Gastropoda: Pulmonata) (http://www.marinespecies.org/aphia.php?p=taxdetails & id=881469). Earlier, Adema et al. (1992) reported the presence of two types of haemocytes in *Achatina fulica* i.e., round cells

and spreading cells. The authors also examined 5 other species of gastropods for the haemocytes under light microscope. The detail classifications, structures and functions of circulating haemocytes of *Lissachatina fulica* were not clearly described.

The present study aims to identify and categorize different circulating haemocytes in the haemolymph of *Lissachatina fulica* (Bowdich, 1822) and their responsiveness against the spores of *Rhizopus* sp and *Aspergillus* sp. The characteristic responses of the various haemocytes against these fungal spores are studied using light and scanning electron microscope in different time lag period. The morphology and morphometry of the circulating haemocytes of both control and fungal spore challenged snails are also compared here. The hypothesis of the study is that specific sub-population of circulating immunocytes would exhibit specific defense responses against specific antigen.

Material and methods

Sampling and rearing of experimental animals

Total 120 active, healthy *Lissachatina fulica* were collected from the field around Kolkata during rainy season (June-July). The snails were kept in a wooden box and acclimatized for five days, providing with leafy vegetables and spraying water throughout the study period and controlled temperature (27°C ± 2° C) and humidity (90 ± 10 %) to minimize seasonal stress. The *Rhizopus* was collected from the moist used tea leaves and the *Aspergillus* was collected from the fungi infested breads. These fungi were maintained in two separate glass jars and maintained a moist condition by frequently spraying water.

Work design of the study and collection of the haemolymph

Total 100 snails of similar size group (average shell length 6.75 ± 0.25 cm) were selected for the study and were divided into four wooden

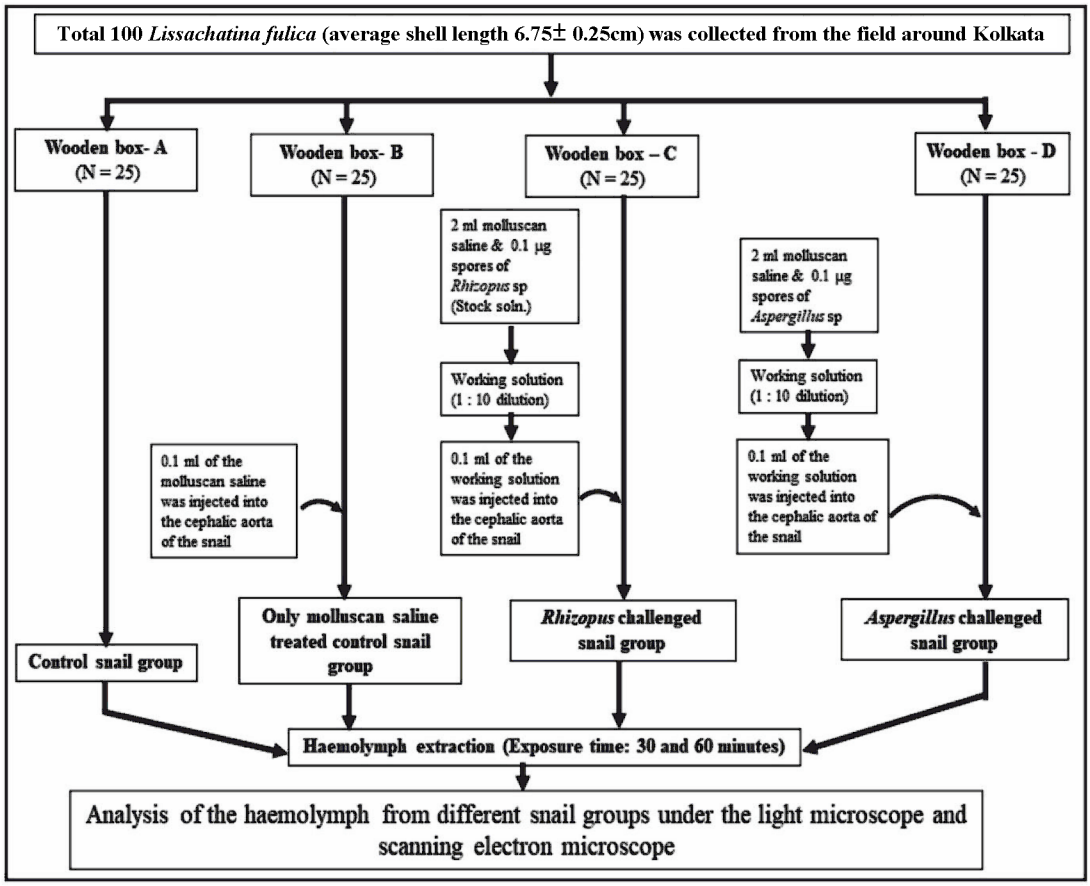


Fig. 1 The work design of the study to investigate the immune responses of the various circulating haemocytes in *Lissachatina fulica* challenged the spores of *Rhizopus* sp and *Aspergillus* sp.

boxes, marked as the control snail group (N = 25), only molluscan saline treated control snail group (N = 25), *Rhizopus* challenged snail group (N = 25) and *Aspergillus* challenged snail group (N = 25) (Fig. 1).

The spores of these two fungi were collected into individual petri dish by simply scrapping the surface of the corresponding tea-leaves and bread with sharp scalpels. An individual stock solution for each fungal spores was prepared (0.1 mg spores: 2 ml molluscan saline) (Adema et al., 1992). A working solution was prepared with 1:10 dilution with molluscan solution from the respective stock solutions and kept into individual glass vial and marked properly (Table 1). The working solution (about 0.1ml)

of the *Rhizopus* spore and *Aspergillus* spore was injected using commercially available insulin syringe into the cephalic aorta of the individuals in both experimental groups; *Rhizopus* challenged snails and *Aspergillus* challenged snails respectively.

The haemolymph was collected from the live individuals of the four snail groups by using individual sterile insulin syringe following a standard method (Oakes, 2008; Bakry, 2009). The needle was inserted into the visceral aorta of the individuals (N = 5) and the desire haemolymph was collected from the four experimental snail groups by slow pulling of the plunger of the syringe on the 30 and 60 minutes of the fungal spore administration (Fig. 1). The haemolymph from all the four snail groups were used to study the haemocytes and their morphological properties under the light microscope (Nikkon 50i) and the scanning electron microscope (Fei, Quanta 200).

Light microscopic study

For the morphological and morphometric studies of the haemocytes both the thick and thin smears of haemolymph were prepared, stained with the Haematoxylin-Eosin and Giemsa stain and examined under light microscope. The analysis of a single drop was done here with two vital stains such as the Janus green and Methylene blue (0.1 %). In the single drop analysis, a drop of haemolymph was kept on a clean glass slide (25 mm × 75 mm) and a tiny drop of a vital stain was pour on the haemolymph drop using a fine needle tip covered by a watch glass and the whole setup was kept in a cold (4°C) chamber for 15 minute to settle as well as staining of the suspended cells. The stage micrometer scale (0.01mm, Erma; Tokyo) and ocular micrometer scale (1 ocular division = 4.35 µm in 400X magnification) were used to measure cellular parameters under light microscope.

Table 1. Different immunogenic components (fungal spores) inoculating into *Lissaachatina fulica*.

Immunogenic components (Fungal spores)	No. of spores in solution		State of immunogen interaction	Time of exposure (Minutes)	Stains
	Stock solution (0.1 mg)	Working solution (ten times dilution)/ (1 ml)			
Spores of *Rhizopus* sp	~ 4250 - 4300	~ 214	*in vivo* & *in vitro*	30 & 60	Jenus green
Spores of *Aspergillus* sp	~ 4190 - 4210	~ 210	*in vivo* & *in vitro*	30 & 60	Methylene blue

Differential count and viability assay of the haemocytes

The population of different haemocytes was evaluated by the thin smear analyses of the haemolymph of both the control and the spore challenged snails. These all smears were stained and more than 200 cells were examined under light microscope with 400X magnification. The haemocytes were categorized depending on relative cell size, spreading properties, cytoplasmic variations, phagocytic ability as well as their staining properties of both the nucleus and cytoplasm.

Additionally, the haemolymph was incubated in Trypan Blue (0.1%) for five minute and counted the dead cells to determine cell viability by Trypan Blue exclusion assay (Accorsi et al., 2013).

Scanning Electron Microscopy (SEM) of the haemocytes

For the ultrastructural studies of the cell surface of the haemocytes, some of the collected haemolymph of the four groups of the *Lissachatina fulica* were used to make the individual thin smears of the haemolymph. An individual thin smear was made on a small clean glass cover slip (5 mm in diameter). After air-dried the smears, all the smear containing cover slips were fixed with Karnovsky's fixative (pH 7.2) for 30 minutes. The smears were washed with phosphate buffer solution (pH 7.4) and dehydrated with ascending grade of the ethanol. The samples were coated with gold using S150 Sputter Coater and examined under SEM (Fei, Quanta 200).

Results

In the present study, it is observed that there are no such significant differences of the haemolymph constituents between the individuals of the control snail group and the only molluscan saline treated snail groups. The following sections are dealt with the comparative morphological characteristics of the haemocytes between the control snail group and two fungal spore challenged snail's groups.

Morphology and morphometry of the haemocytes

The snail possesses three distinct populations of haemocytes on the basis of their size, spreading properties and cytoplasmic variations of the cells. The cell populations are named as Group I (Gr-I) cells, Group II (Gr-II) cells and Group III (Gr-III) cells (Fig. 2). The comparative morphological (Figs. 3"5) and morphometric analysis of different haemocytes along with their staining properties are tabulated in Table 2.

The Gr-I cells are characterised by small sized, round cells (Figs. 3 and 4A). The size of the cell is 12.92 ± 0.09 μm in diameter and its population size is 16 ± 5 % in the circulation. These cells are either non-spreading or very slightly spreading in nature and easily distinguished after vital stain.

The Gr-II cells are pseudopod forming, spreading cells with various sizes. The Gr-II cells are further categorised into two classes depending on cytoplasmic granulations; agranulocyte (ag) (Fig. 4B) and granulocyte (Figs. 4C-D). The agranulocytes (16.92 ± 0.09 μm in diameter) are composed of large nucleus with agranulated cytoplasm (Figs. 4B and 4A). The agranulocyte is slightly spreading cell and the population was 12 ± 3 % in circulation. The granulocytes are characterized by granulated cytoplasm and having two sub-classes; small granulocyte (sg) cells (Fig. 4C) and large granulocyte (lg) (Figs. 4D and lg, in 5A). The small granulocytes (11.35 ± 0.38 μm in diameter) possess circular nucleus, occupies most of the cellular area and was surrounded by thin rim of cytoplasm (Fig. 4C). The large granulocyte (15.44 ± 2.55 μm in diameter) contains large nucleus with no definite shape. The nucleus of large granulocytes is located either acentrically or eccentrically in the cell. The cytoplasm of the large granulocytes consists of distinct small vacuoles (Fig. 4D). The population size of small and large granulocytes is 14 ± 4% and 33 ± 3 % respectively in circulation.

The Gr-III cells are more variable in their shape and size. The cytoplasm and nucleus of these cells are identical in staining characters and fibrous in nature. Although the whole cell body appeared as typical magenta or bluish-purple colour after Giemsa staining (Table 2). In case of haematoxylin and eosin stain the total cell body of these cells were pinkish with a very little blue portion of nucleus. The Gr-III cells are categorized into three subtypes: Type-A Gr-III (Gr-IIIa) cells, Type-B Gr-III (Gr-IIIb) cells and Type-C Gr-III (Gr-IIIc) cells and are designated as "Pleomorphic fibrillar cells" (Figs. 4E-G and 5B-D). The Gr-IIIA cell (15.70 ± 2.16 μm in diameter) is circular in shape and consists of prominent deeply stained circular or kidney shaped nucleus (Figs. 4E and 5B). The Gr-IIIB cells (20.14 ± 4.35 μm in diameter) have no definite shape and comparatively larger than Type-A Gr-III cells. The cell cytoplasm of Type-B Gr-III cell is fibrous with indistinct nucleus (Figs. 6F and 5C). The Type-C Gr-III cell (25.1 ± 8.70 μm in diameter) is larger than Type-A Gr-III and Type-B Gr-III cells. The thin fibrous cytoplasm contains very indistinct fibrous nucleus (Figs. 4G and 5D). In the circulation the population size of three subtypes (Type-A, Type-B, and Type-C) of Gr-III cells were 13 ± 2 %, 10 ± 2 %, and 6 ± 3 respectively.

The cell viability in the undiluted haemolymph is unchanged up to 45 minutes of extracted haemolymph.

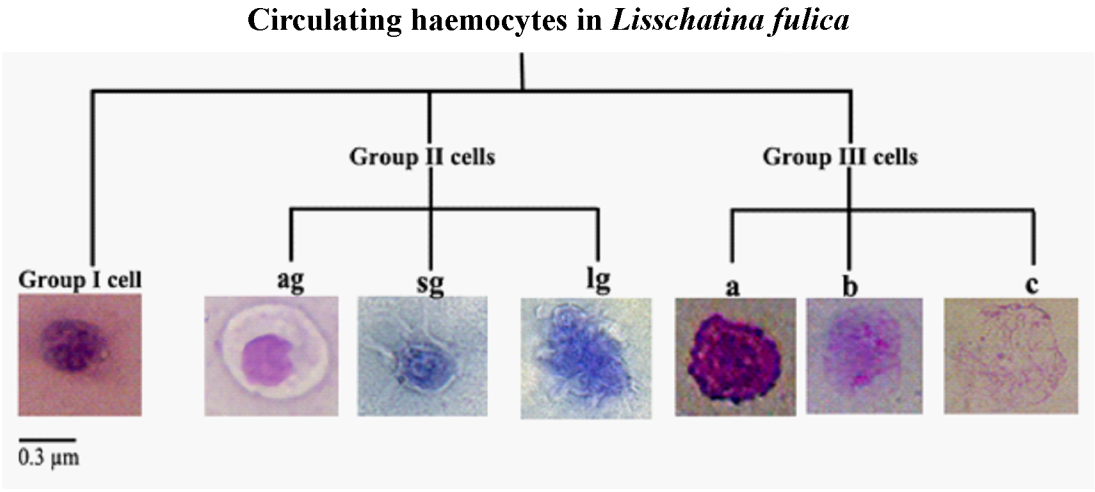


Fig. 2 An illustrative tree of different circulating haemocytes in the haemolymph of the control *Lissachatina fulica.* There are three major cell populations as Group I cells, Group II cells and Group III cells. The Group II cells include three sub-classes as the agranulocyte (ag), small granulocyte (sg), large granulocytes (lg). The Gr-III cells consist of three subclasses as Type-A Group III (a) cell, Type-B Group III (b) and Type-C Group III (c) cell.

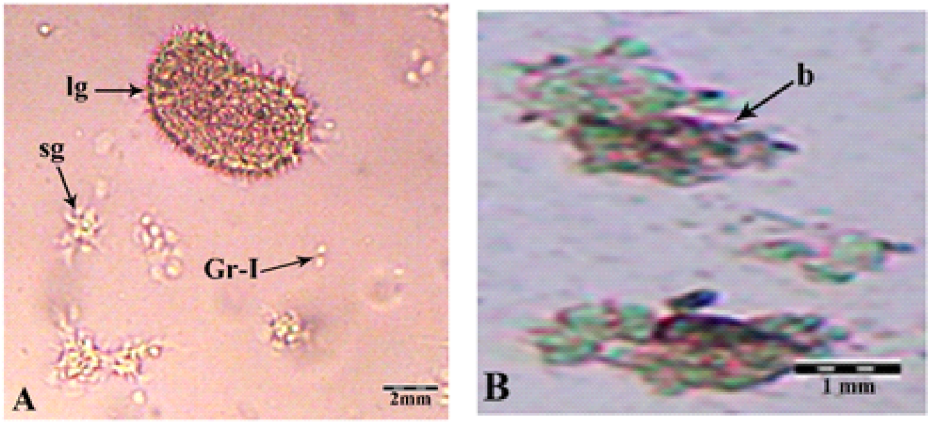


Fig. 3 Circulating haemocytes of the control *Lissachatina fulica* after single drop analysis with Jenus green stain. **A,** Group I (Gr-I) cells, small granulocyte (sg) and large granulocyte (lg). **B,** Type-B Group III cell (b).

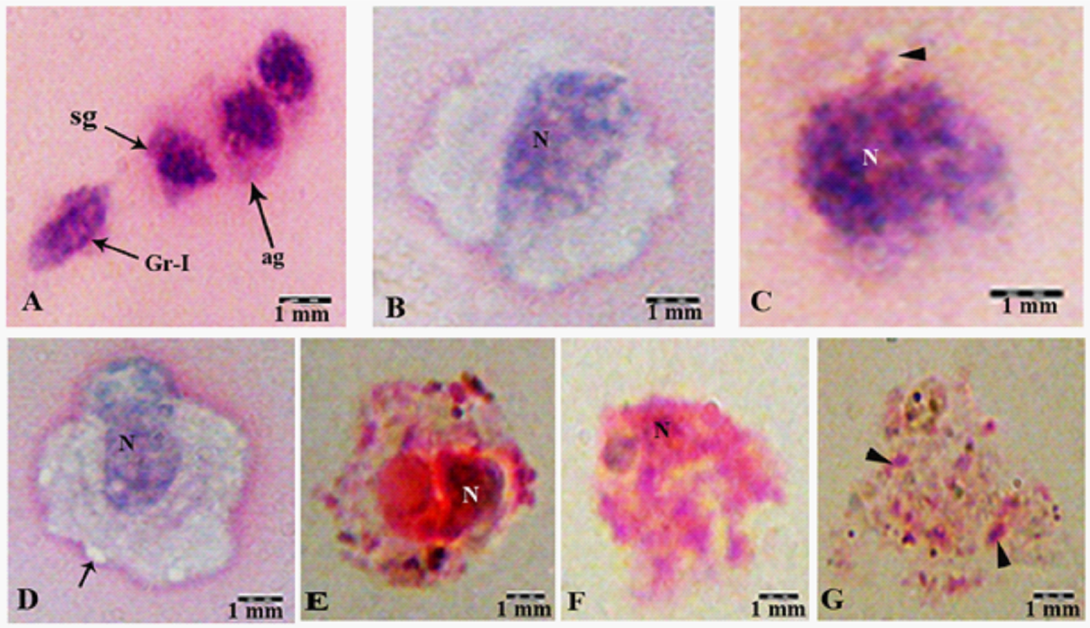
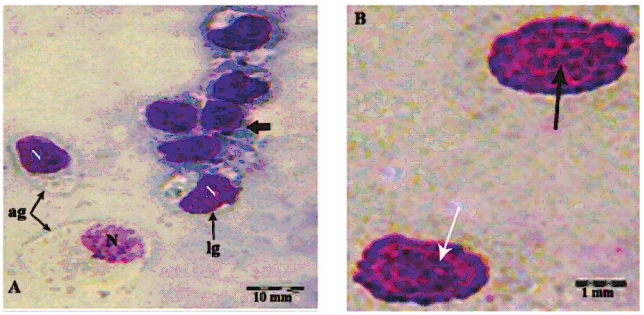


Fig. 4 Thin smear of haemolymph of the control *Lissachatina fulica* stained with haematoxylin-eosin. **A,** Gr-I cells with thin rim of cytoplasm. **B,** Agranulocyte with agranular cytoplasm. **C,** Small granulocyte with short pseudopodia (arrow head). **D,** Large granulocyte with many vacuoles (arrow) in granular cytoplasm. **E,** Type-A Gr-III cell with distinct kidney shaped deeply stained nucleus (N). **F,** Type-B Gr-III cell with indistinct nucleus in fibrous cytoplasm. **G,** Type-C Gr-III cell with fibrous nucleus and cytoplasm. Staining properties of both nucleus and cytoplasm are same. Nucleus is not clearly noticeable and indicated by deeply stained scattered granules (arrow head) in the fibrous cytoplasm. Ag = agranulocyte, Gr-I = Group I cell, sg = small granulocyte, N- nucleus.



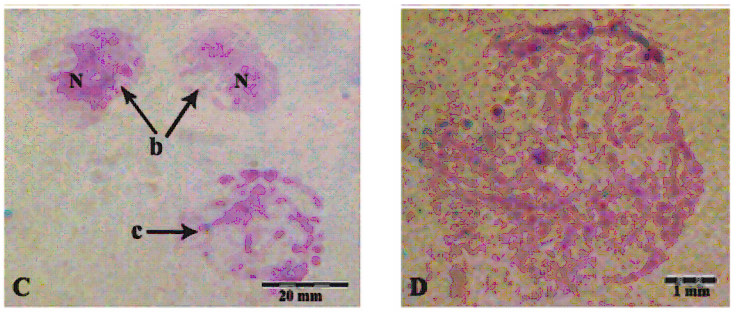


Fig. 5 Thin smear of haemolymph of the control *Lissachatina fulica* stained with Giemsa stain. **A,** Agranulocytes (ag), large granulocyte (lg) and cell nodule (bold arrow) of different haemocytes. **B,** Type-A Gr-III cell with prominent nucleus (arrows). **C,** Type-B (b) and Type-C (c) Gr-III cells with fibrous body. **D,** A typical Type-C (c) Gr-III cell with highly fibrous body. Nuclear materials are very indistinct. Staining properties of both nucleus and cytoplasm are same. lg = large granulocyte, N = nucleus.

Table 2. Morphology and morphometry comparisons of different haemocytes in the haemolymph of the control *Lissaachatina fulica*.

Cell types	Cell morphology	Cell population (%)	Nucleus morphology	Staining (Giemsa) properties		Cytoplasm	Cell diameter (µm)
				Cytoplasm	Nucleus		
1. Gr-I cell	Round with smooth cell membrane	16 ± 5	Circular, occupy most of the cytoplasm	Light pink	Blue	Small and agranular	12.92 ± 0.09
2. Gr-II cells							
ag	Semi-circular to oval with irregular cell membrane	12 ± 3	Circular or elliptical, centric or acentric	Light pink	Bluish pink	Large and granular	16.92 ± 0.09
sg	Circular with irregular cell membrane	14 ± 4	Circular, mostly occupy the cell	Light pink	Blue	Small and granular	11.35 ± 0.38
lg	Ovoid or circular with rough cell membrane	33 ± 3	Circular or elliptical, acentric or eccentric	Light pink	Blue	Large and densely granular	15.44 ± 2.55
3. Gr-III cells							
a	Mostly circular with smooth cell membrane	13 ± 2	Round, homogeneous and centric	Magenta	Deep blue	Moderate, homogeneous and densely granular	15.70 ± 2.16
b	No definite shape with lightly stained thin cell membrane	10 ± 2	Fibrous with dark coarse granules	Magenta	Magenta	Fibrous and granular	20.14 ± 4.35
c	No definite shape with very lightly stained cell membrane	6 ± 3	Fibrous with dark very small granules	Magenta	Magenta	Highly fibrous and granular	25.1 ± 8.70

Note: ag, Agranulocyte; lg, Large granulocyte; sg, Small granulocyte; a, Type A Gr-III cell; b, Type B Gr-IIII cell; c, Type C Gr-III cell.

Responses of the haemocytes against the spores of *Rhizopus*

Repeated single drop analyses of haemolymph with Janus green stain showed that after 30 minute of spore inoculation, the Gr-I cell together with other haemocytes form numerous small cell masses (Figs. 6A-E). The Gr-II cells produce a large number of pseudopods (Fig. 6A). The nodules are frequently distributed as small cell mass in the haemolymph (Figs. 6A-B). The Gr-III cells function as thin adhesive mats with very rough surface (Figs. 6C-E). The nuclear morphology, stainability, granulations of haemocytes between control and infected snails were almost same.

After 60 minute of inoculation, free Gr-I cells are not clearly seen in haemolymph. Some

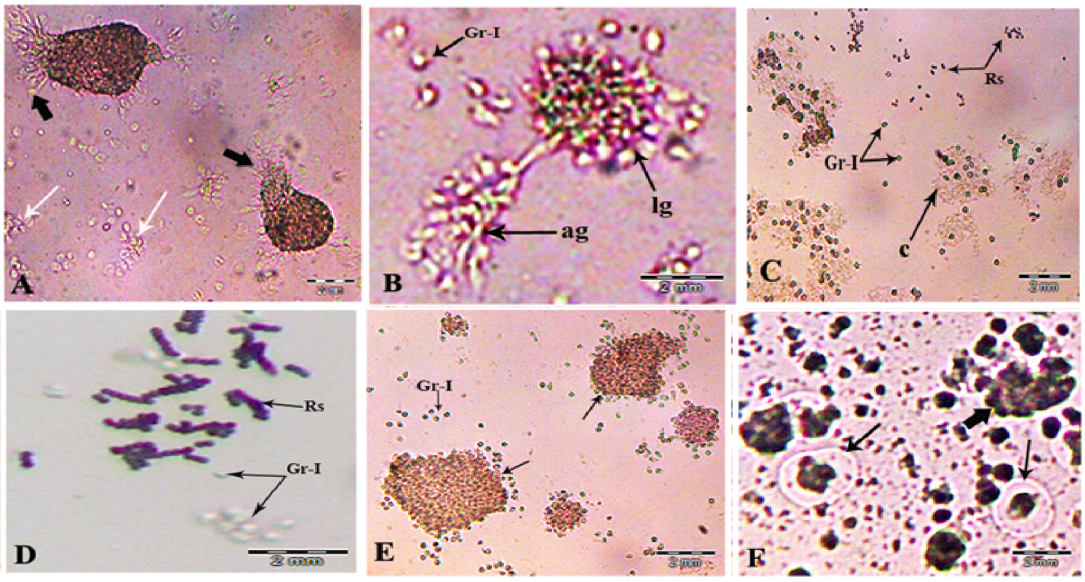


Fig. 6 Different haemocytes of the *Lissachatina fulica* after 30 minutes (A-E) and 60 minutes (F) time lag of inoculation by spores of *Rhizopus* sp (single drop analysis with Janus green stain). **A,** Large granulocytes showing numerous pseudopods (bold arrow). Aggregation of Gr-I cells and formation of small cell nodules (white arrow). **B,** Cell-cell interaction between Gr-II haemocytes. **C,** Spores are arrested on Type-C (c) Gr-III cells. Note some Gr-I cells on the Type-C cell. **D,** Mass of Gr-I cells and spores (Rs). **E,** Aggregation of numerous Gr-I cells with Type-B Gr-III cell. F, After 60 minutes phagocytic haemocytes displaying complete engulfment (thin arrows) of the spore. Note the lysis of the cell nodule (bold arrow). Ag = agranulocyte, Gr-I = Group I cell, lg = large granulocyte.

spores were completely encircled by some Gr-II cells (Fig. 6F). The changes of Gr-III cells are not found. The cell nodules are degraded into small fragments. The cell diameter of different haemocytes in the R*hizopus* challenged snails are slightly increased as Gr-I (12.95±0.02 μm in diameter), agranulocyte (16.97 ±0.01 μm in diameter), small granulocyte (11.36±0.01 μm in diameter), large granulocyte (15.45 ± 0.02 μm in diameter), Type A Gr-III cell (15.79 ± 0.1 μm in diameter), Type B Gr-III cell (20.19 ± 0.15 μm in diameter), and Type C Gr-III cell (25.17 ± 0.3 μm in diameter). These cell sizes of the various haemocytes are characteristically comparable with the haemocytes in the haemolymph of the control snail group (Table 2).

Response of haemocytes against the spores of *Aspergillus*

There are some morphological variations of the haemocytes after drop analyses with Methylene blue stain after 30 minutes of inoculation (Figs. 7A-D). The Gr-I cells form large cell mass along with other haemocytes (Fig. 7A). The Gr-II cells encircle the spores of *Aspergillus* through either tubular long pointed filopodia (Fig. 7B) or short blunt wing like lobopodia (Fig. 7C). Any significant morphological changes of the Gr-III cells are not observed.

After 60 minutes of inoculation, it is observed that some spores are completely encircled by large granulocyte cells (Fig. 7D). The cell diameter of different haemocytes in the infected snail were slightly increased as the Gr-I cell (12.94 ± 0.01 μm in diameter), agranulocyte cell (16.92 ±0.1 μm in diameter), small granulocyte (11.35 ± 0.02 μm in diameter), large granulocyte (15.44 ± 0.02 μm in diameter), Type A Gr-III cell (15.79 ± 0.2 μm in diameter), Type B Gr-III cell (20.18 ± 0.1 μm in diameter), and Type C Gr-III cell (25.17 ± 0.4 μm in diameter).

A comparative morphometrical features of the various haemocytes in the control snail group and two different fungal spore (*Rhizopus* and *Aspergillus*) challenged snail groups are graphically represented here (Fig. 8). It is

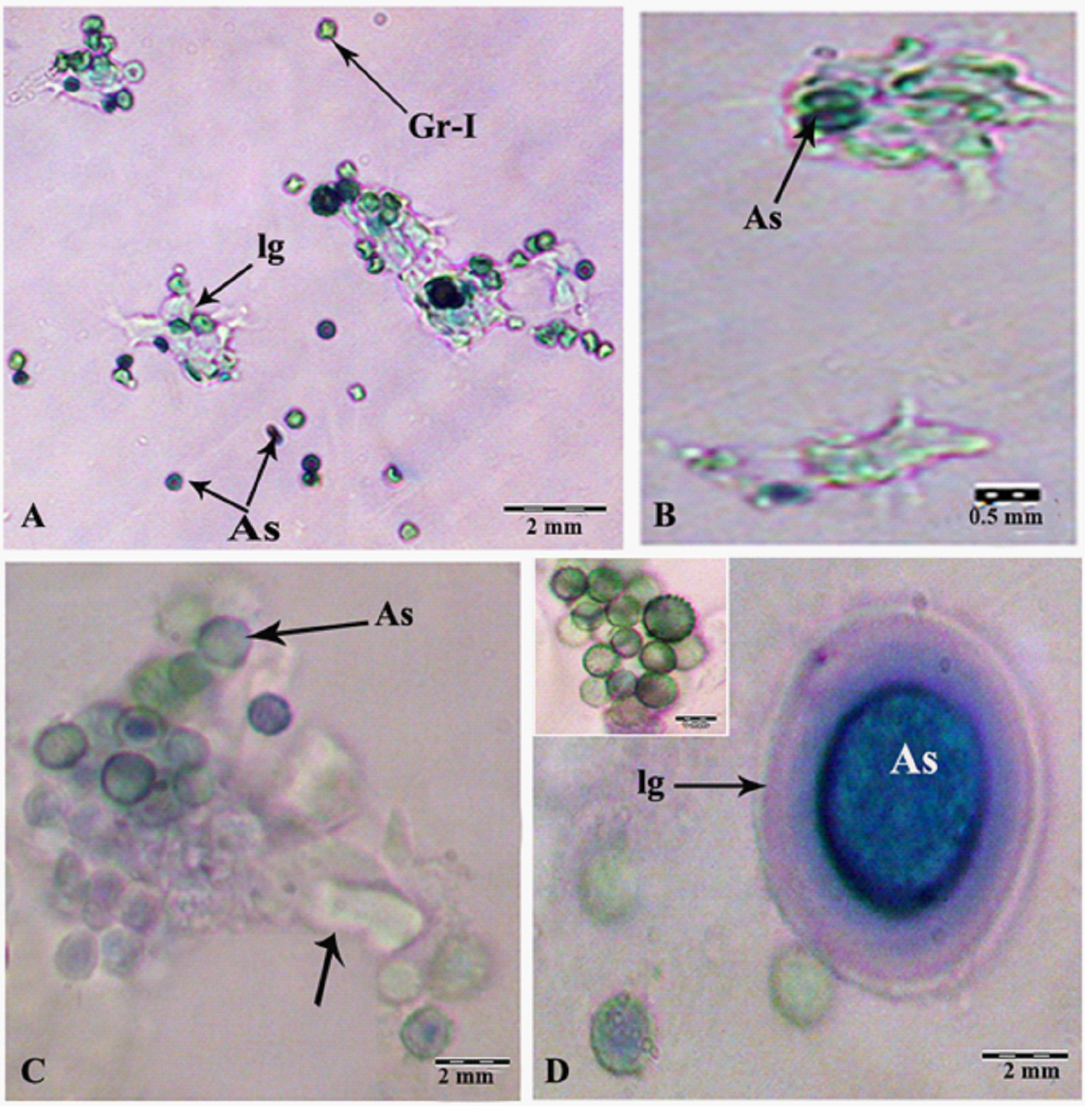


Fig. 7 Responses of haemocytes of the *Lissachatina fulica* after 30 minutes (**A-C**) and 60 minutes (**D**) time lag of inoculation by the spores of *Aspergillus* sp (Methylene blue stain). A, haemocytes involve in formation of nodule and in capturing of spores. **B,** Large granulocytes capture the spores (As). C, Wing like pseudopodial extensions (arrow) of large granulocyte. **D,** A large granulocyte (lg) completely engulfs a spore (As) of *Aspergillus*. Enlarged views of surface structure and staining property of spores are shown in shed. Gr-I = Group I cell.

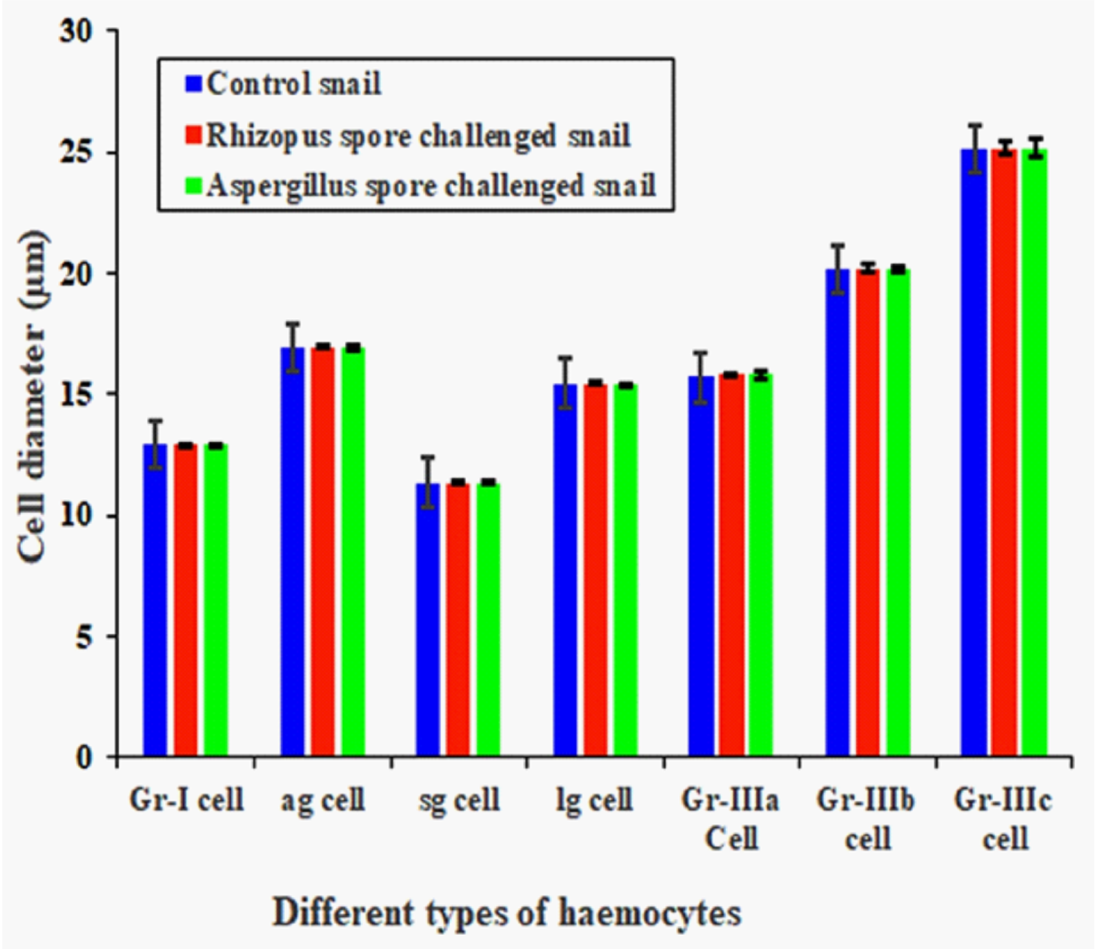


Fig. 8 Graphical comparison of the cell size (cell diameter) of the different haemocytes in the haemolymph of the control snail groups and different fungal spore challenged snail groups of *Lissachatina fulica.*

Table 3. Comparative cell diameter of different haemocytes in the haemolymph of control and two fungal spores (*Rhizopus* sp and *Aspergillus* sp) challenged *Lissaachatina fulica*.

Types of the haemocytes	Control snail	*Rhizopus* spore challenged snail	*Aspergillus* spore challenged snail
Gr-I cell	12.92 ± 0.09	12.95 ± 0.02	12.94 ± 0.01
ag cell	16.92 ± 0.09	16.97 ± 0.01	16.92 ± 0.01
sg cell	11.35 ± 0.38	11.36 ± o.o1	11.35 ± 0.02
lg cell	15.44 ± 2.55	15.45 0.02	15.44 ± 0.02
Gr-IIIa Cell	15.7 ± 2.16	15.79 ± 0.01	15.79 ± 0.2
Gr-IIIb cell	20.14 ± 4.35	20.19 ± 0.15	20.18 ± 0.1
Gr-IIIc cell	25.1 ± 8.7	25.17 ± 0.3	25.17 ± 0.4

Note: ag, Agranulocyte; lg, Large granulocyte; sg, Small granulocyte; Gr-IIIa, Type A Gr-III cell; Gr-IIIb, Type B Group-III cell; Gr-IIIc, Type C Gr-III cell.

observed that the spores of the *Rhizopus* sp have more immunogenic effects than that of the *Aspergillus* sp (Table 3).

SEM observations

The haemocytes in the control haemolymph are oval to semi-circular in shape with concavo-convex surface (Figs. 9A-F). The Gr-I cells are round to oval in shape and smaller in size than other cell groups. The Gr-II cells are with distinct concave and convex surfaces. The concave surface of Gr-II cells is rough with numerous marks of budding pseudopods (Fig. 9C). The whole concave surface of the Gr-II cells is appeared as honey combs like structure. The convex surface of the cells is almost smooth (Fig. 9B). In some cases, the Gr-II cells form short, tubular lobopodia with smooth surface (Fig. 9D). The Type-A Gr-III (Fig. 9E) and Type-B Gr-III (Fig. 9F) cells are commonly found in the smear.

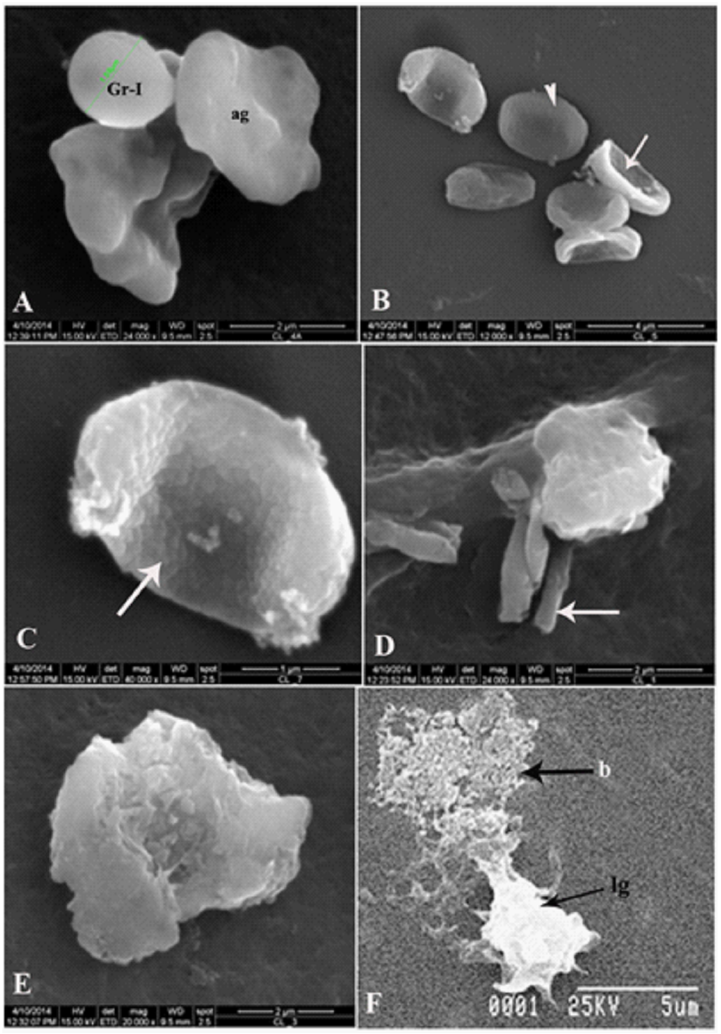


Fig. 9 Scanning electron micrographs of the haemocytes in the hemolymph of the control *Lissachatina fulica*. **A,** Cell nodule consists of different haemocytes. **B,** Large granulocyte showing smooth convex (arrow head) surface and rough concave surface (arrow). **C,** Magnified view of the concave surface (arrow) (honey comb like surface) of a large granulocyte. **D,** Lobopodia like pseudopods (arrow) of a phagocytic cell. E, Type-A Gr-III cell with uneven cell surface. F, Type-B Gr-III cell (b) adhere with a large granulocyte. ag = agranulocyte, lg = large granulocyte, sg = small granulocyte.

Responses of the haemocytes against the spores of *Rhizopus*

After 30 minute of spore inoculation, haemocytes show various structural modifications of pseudopods (Figs. 10A-K). Gr-I cells are found with no more modification. A typical whip like pseudopod is formed by Gr-II haemocytes. The cell is attached to the substratum by thick, sticky membranous structure (Fig. 10A). The filopodia (Figs. 10B, D, H, I, J), lobopodia (Figs. 10B, D, J) and axopodia (Fig. 10C) are characteristic pseudopodial modifications of Gr-II haemocytes.

After 60 minutes of inoculation, the responses of haemocytes are reduced (Figs. 11A-D). Gr-II haemocytes display very short pseudopodia (Figs. 11A-B). The Type-C Gr-III cells are very prominent in the cell nodules (Figs. 10C-D). The spores of *Rhizopus* are lightly attached on the haemocytes (Figs. 11C-D).

Responses of the haemocytes against the spores of *Aspergillus*

After 30 minute of inoculation, Gr-I cells are mostly found in nodules. Gr-II cells generate long tubular drum-stick like pseudopods from the concave surface of the cell (Figs. 12A-B). The free end of the pseudopods possesses a typical small circular head/knob (Fig. 12B). In some cases, the phagocytic haemocytes are changed with a cell surface ornamentation (Fig. 12C). The Gr-III cells displaying surface ornamentations composed of numerous small pits (Figs. 12 C-D). The marginal part of each of these membranous pits consists of short upwardly pointed spines (Fig. 12D).

After 60 minutes of inoculation, the haemocytes become solitary (Figs. 13A-D). The roughness of cell surface is increased than that of the 30 minute of lag periods (Figs. 13A-B). In haemocytes, the ability of pseudopod formation is decreased (Figs. 13A-C). The nodules are small in size but more compact in nature (Fig. 13D).

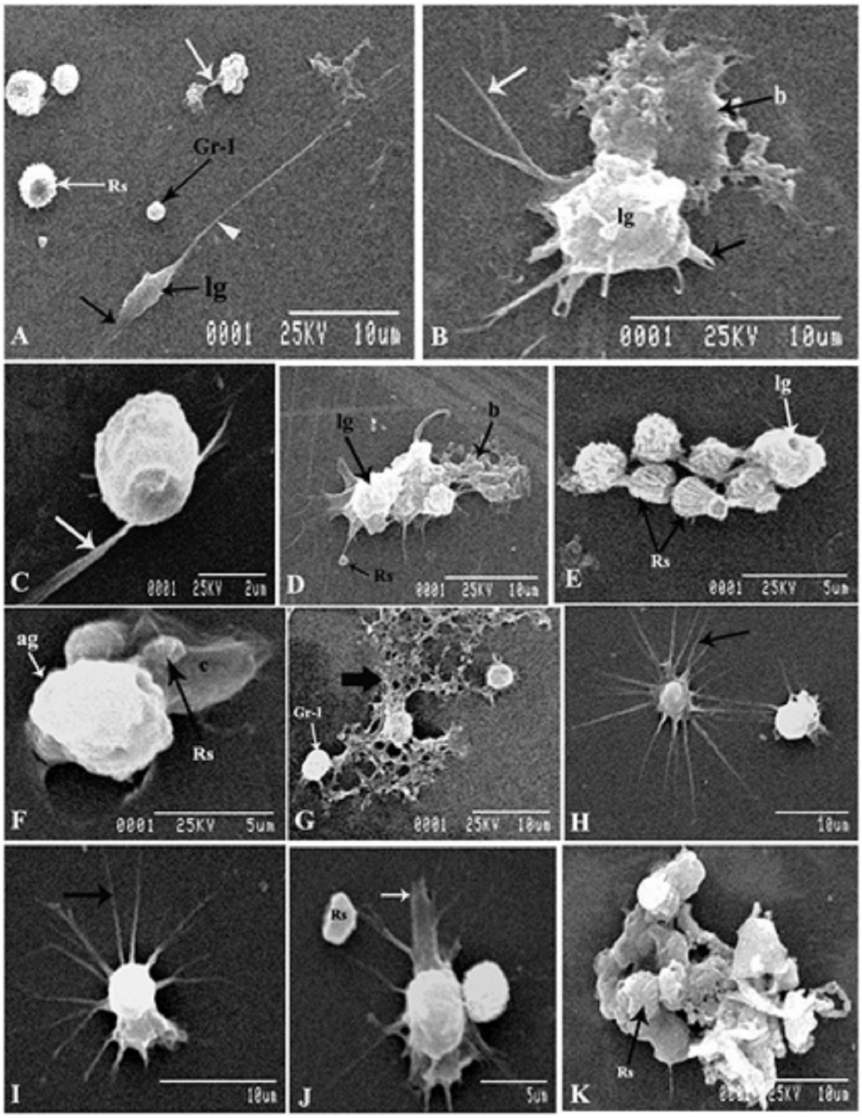


Fig. 10 Surface ultrastructure of the haemocytes of the *Lissachatina fulica* after 30 minutes inoculation of spores of *Rhizopus* sp. **A,** Whip like pseudopodial extension (white arrow head) of Gr-II cell. The cell body adheres to the substratum by a thin membranous structure (black arrow). Note the pseudopodial connection (white arrow) between cell nodules. **B,** Lobopodial (black arrow) and filopodial (white arrow) extension around the cell surface of Gr-II cell. C,

Fan like axopodia (arrow) formation of a Gr-II cell. **D,** Spore (Rs) is arrested by the filopodia of a haemocyte in a cell nodule. **E,** Phagocytic Gr-II cells arrest many spores in a file. **F,** Spores are wrapped by Gr-IIIC haemocyte. **G,** Mat or bed formation (bold arrow) by the aggregation of many Gr-IIIC cells and facilitates to hold the other haemocytes and spores. H-I, Typical tubular pointed filopodia of Gr-II phagocytic cells. **J,** Typical blunt lobopodia (white arrow) formation of Gr-II cell. **K,** Nodule formation of haemocytes and spores of *Rhizopus*. Gr-I = Group I cell, Gr-II = Group II cell, lg = large granulocyte, sg = small granulocyte, Rs- spore of *Rhizopus*.

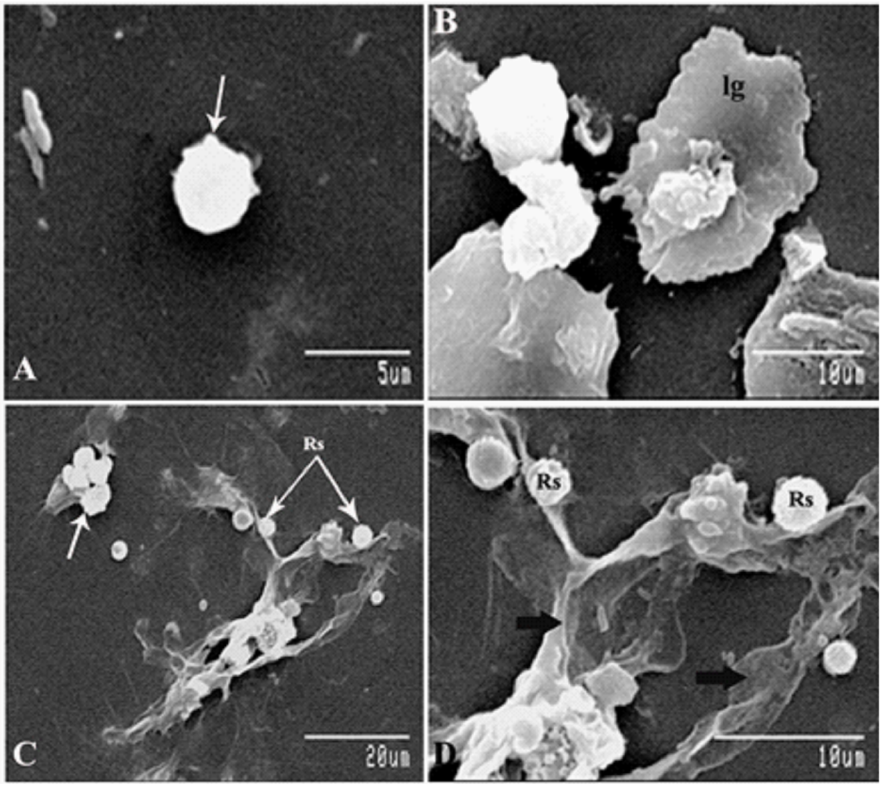


Fig. 11 The haemocytes of *Lissachatina fulica* after 60 minutes interaction with the spores of *Rhizopus* (Rs). **A,** haemocytes reduce their digitations around the cell surface (arrow). **B,** Weak cell-cell connections between haemocytes. **C,** Small nodule of haemocytes and thin cytoplasmic mat formation of Type-C Gr-III haemocytes. **D,** Magnified view of a portion of Fig. C. lg = large granulocyte.

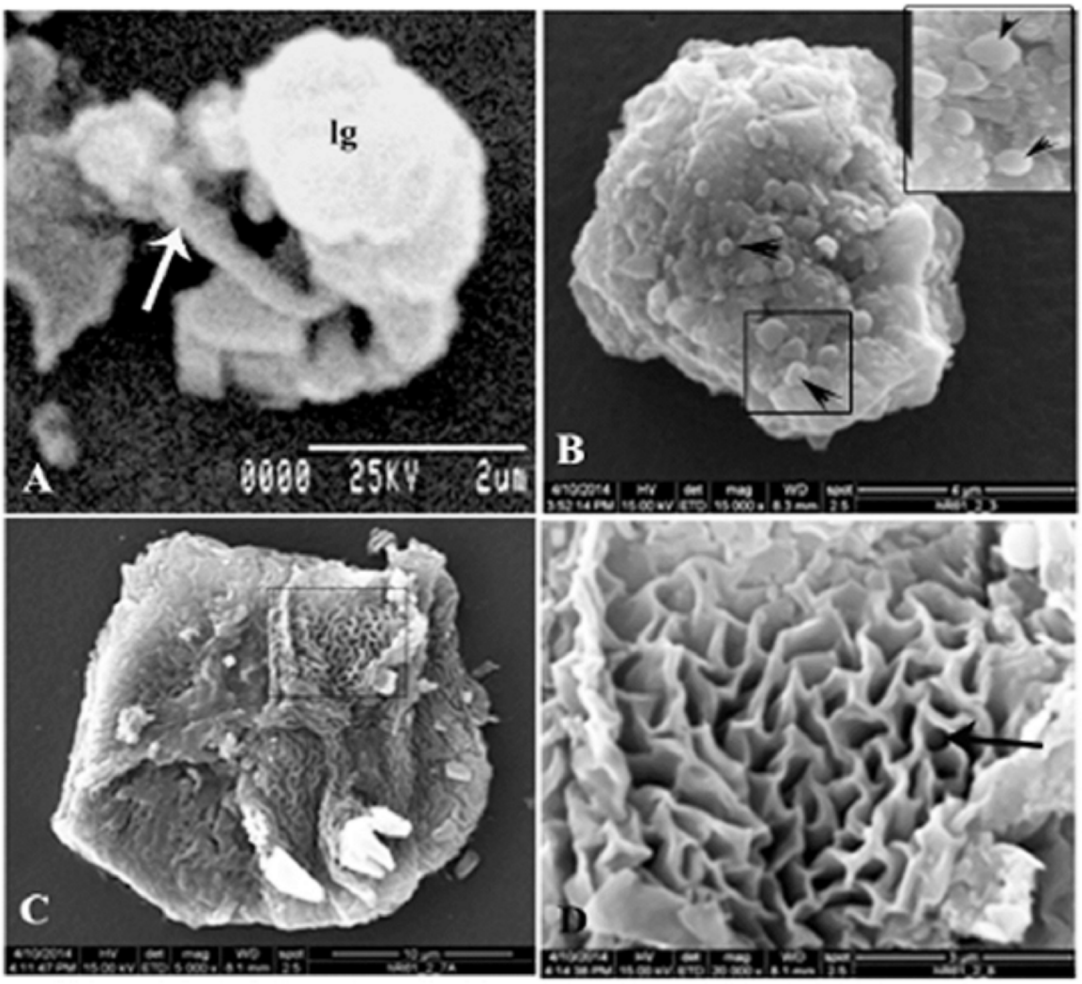


Fig. 12 Ultrastructural modifications of haemocytes of *Lissachatina fulica* after 30 minutes interaction with *Aspergillus* sp. **A,** Drum stick like pseudopodial extension (arrow) from a Gr-II phagocytic cell. **B,** Numerous drum stick like pseudopodial tips (arrow head) on the cell surface of phagocytic haemocytes (Gr-II cell). Magnified view of a marked area is shown in set. Note the knob like head on pseudopods (arrow head). **C,** Folded Gr-IIIC haemocytes displaying characteristic roughness of cell surface. **D,** Enlarged view of a marked area of Fig. C showing numerous small pits with spine like structure (arrow) on the surface of Gr-IIIC cell. lg = large granulocyte.

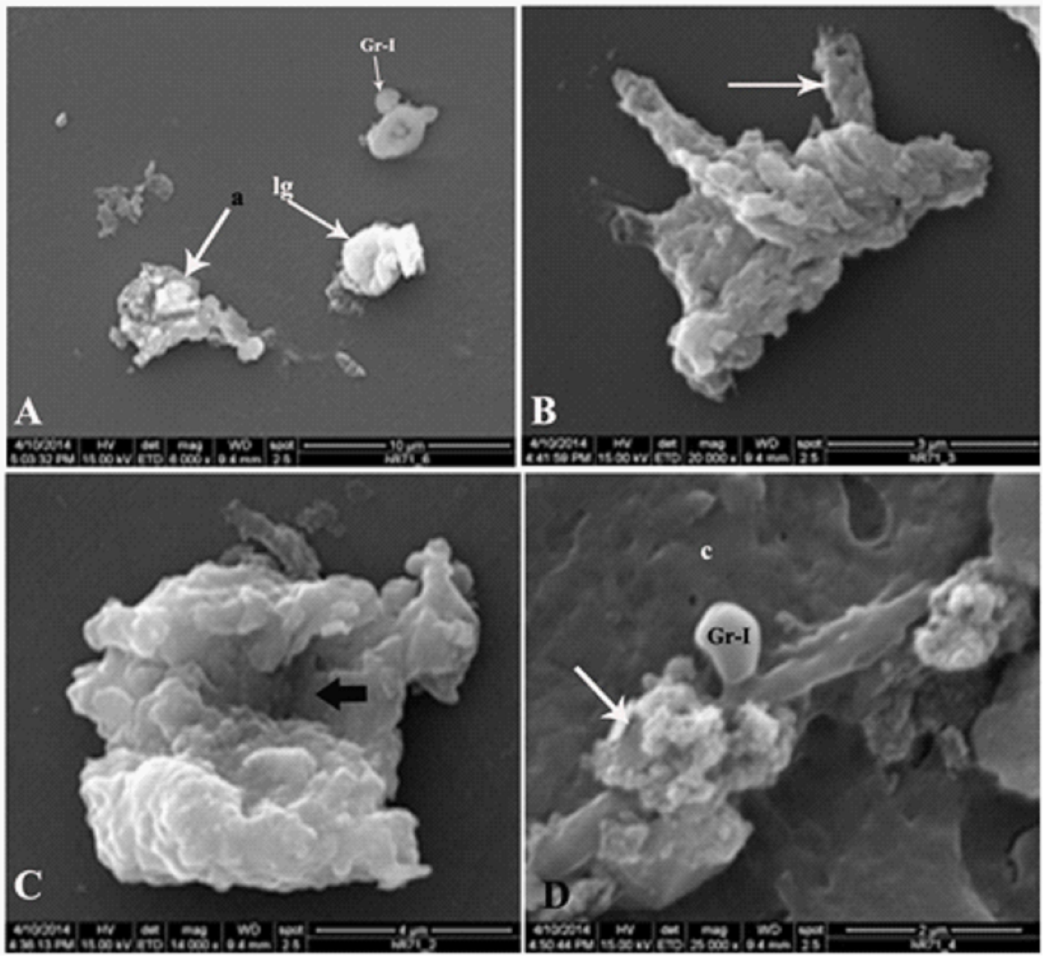


Fig. 13 Modifications of the haemocytes of of *Lissachatina fulica* after 60 minute of interaction *Aspergillus* sp. **A,** Reduction of phagocytic response of haemocytes to the *Aspergillus*. **B,** Characteristic roughness (arrow) of pseudopods of a Group II phagocytic cell. **C,** Declining of pseudopods formation from concave surface (bold arrow) of Group II cell. **D,** Formation of cell nodule (arrow) of different haemocytes on the Type-C Gr-III cell. a = Type-A Group III cell, c = Type-C Group III cell, Gr-I = Group I cell.

Discussion

Immune system differs among molluscan species by the variability of their type of haemocytes (or immunocytes) as well as their respective habitats of the individuals (Adema et al., 1992). In general, aquatic gastropods such as *Lymnaea stagnalis* (Sminia, 1972; Adema et al., 1992), *Biomphalaria glabrata, Bulinus natalensis* (Adema et al., 1992), *Megathura crenulata, Aplysia californica* (Martin et al., 2007), *Indoplanorbis exustus*, *Pila globosa* (Mahilini & Rajendran, 2008), *Bellamya bengalensis* (Ray et al., 2013), *Pomacea canaliculata* (Accorsi et al., 2013; Cueto et al., 2015) possess comparatively small haemocytes than those of terrestrial gastropods as *Helix aspersa*, *Achatina achatina, Achatina fulica* (Adema et al., 1992), and *Helix aspersa maxima* (Adamowicz & Bolaczek, 2003). Two types of haemocytes, large spreading cells and small round cells are reported in terrestrial *Helix aspersa* and *Achatina achatina* (Adema et al., 1992). On the other hand, the aquatic pulmolnates *I. exustus* (Mahilini & Rajendran, 2008), *L. stagnalis* (Adema et al., 1994) exhibit more than two haemocytes as agranulocytes and granulocytes with different subtypes whereas *Planorbarius corneus* exhibit only round and spreading haemocytes (Ottaviani, 1992). The present study records, for the first time, that *Lissachatina fulica* possess three distinct populations of circulating haemocytes. These cell populations exhibit morphological characters as well as their staining properties. Most of the cytological features of the haemocytes of *Lissachatina fulica* exhibit similarities with the different types of haemocytes of other gastropods.

The characteristics of the Group I cell of *Lissachatina fulica* are mostly identical with the round, non-spreading cells as described in many gastropods (Ottaviani, 1992; Adema et al., 1992; Pengsakul et al., 2013). Agranulocytes as well as small granulocytes (pro-immunocytes) show adherence activity and little ability to phagocytosis as previously reported in other molluscs (Mahilini & Rajendran, 2008; Adema et al., 1992; Ray et al., 2013; Cueto et al., 2015; Kumazawa et al., 1991; Mc Cormick-Ray & Howard, 1991). It is assumed that the large granulocytes may be the mature form of small granulocytes. The characteristics of large granulocytes corroborate to the previously described spreading cells or Granulocyte II in other gastropods (Mahilini & Rajendran, 2008; Ottaviani, 1992; Martin et al., 2007; Adema et al., 1992; Cueto et al., 2015; Pengsakul et al., 2013). Group III cells have various functional forms and are described for the first time in this gastropod species. Among the Group III cell types, Type-B is considered as intermediate form of Type-A and Type-C cells. These are all morphological variations of Group III cells and may be recognised to their response against invading antigens. The Type-

A, Type-B, and Type-C of Group III cells may be designated as normal (relax), intermediate and expanded (highly responsive) form respectively.

The defense patterns of haemocytes are considered as one of the parameters in classifying the haemocytes in the haemolymph of *Lissachatina fulica* following Accorsi et al. (2013). The cell morphometry of all cell types of three populations of haemocytes is slightly increased (but not significantly) in fungal spore challenged snails and are almost same against all tested immunogens. Population percentage of the Group I cells and small granulocytes is declined in the circulation of the fungal spore challenged snails, while it is increased for the agranulocytes and large granulocytes in the haemolymph of these experimental snails. In the present study, it is shown that not all haemocytes are reacting against immunogens. Agranulocytes, large granulocytes, Type-A and Type-B of Group III cells are major reactive haemocytes against the invading immunogens and these responses are almost same to all immunogens. Population fluctuations of the circulating haemocytes between control and infected *Lissachatina fulica* corroborate to the population dynamics of uninfected and infected *Biomphalaria alexandrina* (Bakry, 2009). The size and filamentation of Group III cells have increased on exposure of immunogens and assumed that these may promote to capture the particles of the immunogens.

An increase in cell diameter, pseudopod formation and phagocytosis are the major immune response after 30 minute of time lag since inoculation of immunogens. The formation of numerous vacuoles and pseudopodia of Group II cells might be one of the responses against antigens (Bakry, 2009). In the present study, it is observed that the intensity of the pseudopod formation on the cell surface of the various haemocytes and other immunogenic responses of *Lissachatina fulica* after 30 minute of different fungal spores administration is characteristically higher than those of the after 60 minutes of this immunogen administration. It is assumed that such changes of the immunogenic responses of the circulating haemocytes in different time-lag periods may be due to a gradual relaxation of immunogenic responses of these haemocytes after a certain time of haemocyte-immunogen interaction.

The surface ultra-structures of different haemocytes of *Lissachatina fulica* display many similarities as reported in *B. alexandrina* (Bakry, 2009) and *Oncomelania hupensis* (Pengsakul et al., 2013). The surface morphology of the Group III cells is described for the first time, which reveals numerous small pits like structure. Such structure is not reported earlier. Changes in morphology of pseudopodia of haemocytes against antigens

are reported earlier (Martin et al., 2007; Ottaviani et al., 1986; Lapointe et al., 2012). Pseudopodia of large granulocytes show different morphological change against different antigens. The pseudopodia look like long drum stick with a small head in *Aspergillus* inoculated snails, whereas it is pointed filopodia in case of *Rhizopus* inoculated snails. Thus, it is assumed that variations in pseudopod morphology might be specific against specific antigenic response. Surface of the Group III cells changed to short spine-like structure in case of antigen challenged snails. Such change in surface morphology is not specific to antigens.

The haemocytes *Lissachatina fulica* show both non-specific and specific defense responses against the two immunogens (spores of *Rhizopus* sp and *Aspergillus* sp) experimented in this study. The Group I cells, small granulocytes, and Type-B Group III cells are mainly involved in non-specific immune responses that is the reaction of haemocytes are similar against the two immunogens used in the present study. However, immunogen specific changes are distinguished in case of large granulocytes, agranulocytes, Type-A and Type-C Group III haemocytes. It is observed that the cell size of various haemocytes is slightly increased in response to the spores of *Rhizopus* sp than that of the *Aspergillus* sp. It is assumed that the spores of the *Rhizopus* sp having slightly more immunogenic ability than that of the *Aspergillus* sp.

Acknowledgement

The authors are thankful to the principal and all the staffs of Department of Zoology of City College, Calcutta, West Bengal, India. This study was supported by the fund of UGC Major Project of Dr. K. K. Chaki. Authors are grateful to the Laboratory staff of the SEM Unit, Bose Institute, Calcutta and the SEM Unit, University of Burdwan, West Bengal.

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