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Hemocytes Profile of Mud Crab *Scylla tranquebarica* - an Analysis of Light, Phase Contrast and Electron Microscopic Observation

K.Ramlingam[1, *], U.D.Bharathi Rajan[2] N.Sri Kumaran[1] and A. Jaffar Hussian[1]

[1]Centre for Marine Bioprospecting, Department of Marine Biotechnology, AMET University, Kanathur, Chennai 603112, Tamil Nadu, India

[2]Mediclone Biotech Company Private Ltd. Velichai Kolathur (Post), Chennai -603 048

***Corresponding author:** Email: krmbiomed@gmail.com; Mobile No: 9710039843

Abstract

Scylla tranquebarica revealed the presence of prohaemocytes, granular haemocytes, cystocytes, plasmatocytes and podocytes. The diagnostic characteristics of these cells conformed to the descriptions given by earlier authors in decapod crustaceans and insects. The spherule cells, adipohemocytes and oenocytoids have been found to be absent. Anucleate cells have been identified by both light and transmission electron microscopic observations. The discernibility of such anulceated cells added a new dimension to the concept of hemocytes origin and differentiation. The existence of anucleate granulocytes is construed to be a tenable phenomenon and situation considering the stem cells property that they undergo both symmetric and asymmetric divisions to maintan cell population in organisms. The total hemocyte count and the differential count indicated the defence potential of the species.

Keywords: Prohaemocytes, Plasmatocytes, Coagulocytes, Crustacean

INTRODUCTION

In crustacean, the blood cell types viz., haemocytes are classified into freely circulating hemocytes and variety of fixed cells which are found scattered throughout the tissues or localized in the hemopoietic organs or phagocytic organs (Ratcliffe et al., 1985). Five such main cell types include 1) progenitor/ haemoblasts/stemcells, phagocytic granulocytes, plasmatocytes, haemostatis cells viz., cystocytes/ coagulocytes/explosive cells/hyalionocytes, nutritive glycocytes/eleocytes/trophocytes/spherule cells, pigmented cells, etc. In this lineage, recent investigations have added podocytes, lamellocytes into the category of phagocytes.

Invertebrate (crustacean) haemocytes classification in their earlier times was mostly based on structural features, granule size, cell size and the histochemical affinities of contents ect. The established classification system was that of Jones (1962). However several investigators followed their own criteria of identification in different species and sometimes erroneous description were also adduced (Brehelin *et al.,* 1978). The classification of haemocytes by classical dyes was given by ling *et al.,* (2003). The Martin *et al.,* (2003) revealed the importance of N-acetyl D- glucosamine in haemocyte classification. Variable numbers of cell types have been identified due to inhomogenous nomenclature, experimental procedural variations and also due to developmental stages of organisms. Hence, a uniform terminology and classification till date remains uncertain. In the present study, light microscopic, phase contrast and electron microscopic studies on the characterization of 3

different cell types was made with a view to bringing confirmative phenotypic features about the haemocytes in a decapod crabs *Scylla tranquebarica*. In this regard, previous studies have revealed that in decapods crabs, most of the cells belong to the granulocytes category, which are differentiated into small granulocytes and large granulocytes. The hyalinocytes represent scantily in the crabs. In the light of the previous observations, the present study was attempted to characterize the haemocytes profile of the mud crab *Scylla tranquebarica*.

Haemocytes contribute to the defence of crustaceans in multifarious ways. Both in vitro and in vivo studies have demonstrated their diverse immune mechanisms. They include i) the phagocytic response of haemocytes such as granular haemocytes, plasmatocytes, lammellocytes and podocytes, ii) cellular encapsulation (Haemocytes), iii) nodule formation by haemocytes, iv) the granular haemocytes with defence molecules such as clotting serine protease enzymes, a clottable protein coagulogen, protease inhibitors, haemagglutinins (lectins) and antimicrobial peptides. Total haemocyte count (THC) is an indicator of the individual's defence of crustacean species, as the population of circulating haemocytes may indicate whether the host defence system was activated or not (Brehelin et al., 1982). As haemocytes confer protection to disease causing pathogens, their enhancement in circulation as well as the proliferation of cells in the haemopoietic tissues by agents would have positive impact on the part of crustacean's immunity. Hence in the present study, the THC and differential count of hemocytes were determined to understand the immunological potential of the species *Scylla tranquebarica.*

MATERIAL AND METHODS

Specimens of the mud crab *Scylla tranquebarica* were collected at brackish waters of pulicate lake, Chennai. Immediately after collection, the animals were transferred to laboratory and reared in plastic tanks filled with seawater. Intermolt animals were always selected for the study of hemocytes. The stages of molt cycle were identified using the criteria suggested by Drach (1939).

Light Microscopy

1) Method I

Haemolymph was collected by bleeding the last walking leg. The blood was dropped in 3% glutaraldehyde and centrifuged at 2000 rpm for 10 mins. The cells get pelletized and the supernatant was discarded. The cells were prefixed in 3% glutaraldehyde for 1 hour and washed in buffer. The cells were then post fixed in 1 % osmium tetraoxide and washed in buffer. After post fixation the cells were blocked with 2 % agarose. The pellet was dehydrated with ascending series of graded alcohol (50% to 100%) and infiltrated by propylene oxide and epoxy resin. The pellet was embedded in siliconised rubber mould with epoxy resin. The embedded mould was kept in an incubator at 60ºC for 48 hours and then cooled down for sectioning. One micron ultra thin sections were cut through ultra microtome (Leica Ultracut UCT) with glass knife and stained by toluidence blue. After staining, they were gently washed in distilled water. The slides were dried and DPX was added for permanent slide. Photographs were taken using Nikon digital camera (Coolpix 995) with 3.1 Mega Pixel size.

2) Method II

Hemolymph was collected from the crab be cutting the tip of the last walking leg and smeared in microscopic slides immediately and allowed to dry in air. Then, slides were dipped in methanol (100%) for 3-5 min. After fixing them in methanol, they were stained with Giesa stain for 30 to 40 min. Leishman stain was also added to aseparate set of slides. After staining, they were gently washed in distilled water. The slides were then dried and DPX was added for permanent slide. Photographs were taken using Nikon digital camera (Coolpix 995) with 3.1 Mega Pixel size.

Different count (DC)

Differential count was done using the method described by Silva *et al.,* (2002). Differential counts were done in hemolymph smears stained with Giema. One hundred and fifty cells identified from four randomly selected fields were counted per crab. DC was expressed in percentage.

Total haemocyte count (THC)

Total haemocyte count (THC) was done in phase contrast microscope using the method described by Holman et al., (2004). The hemolymph was drawn from the arthrodial membrane present in the base of the walking leg. 10% neutral buffered formalin (NBF) was used as the diluting fluid and the hemolymph was collected using disposable syringe bearing 21gauge needle. The dilution factor was calculated using the following method:

$$\text{Dilution factor} = \frac{\text{(Wt. Of syr+NBF+hemolymph)-(Wt of syr)}}{\text{(Wt. Of syr+NBF+HI) - (Wt. Of syr+NBF)}}$$

Hemolymph was collected along with ice cold NBF in the ratio of 8:2. The solution was mixed well and neubauer RBC counting chamber was used. The calculation for the cells per microlitre was done using the following formula viz.,

$$\text{Cells}/\mu L = \frac{\text{cells counted} \times \text{dilution factor}}{\text{Area} \times \text{thickness}}$$

Six trials of cell counting were made and the mean value with standard deviation is given in the result.

Phase contrast microscopy:

Haemocytes collected for cell count in 10% neutral buffered formalin was observed and photographed. Few drops of hemolymph were added into the interphase between the Neubaur chamber and coverslip and allowed to settle for 3 mins. The

observations of different cell types were made in 40× magnification.

Electron Microscope

Hemolymph was collected by bleeding the last walking leg. The blood was dropped in 3 % glutaraldhyde and centrifuged at 2000 rpm for 10 mins. The cells get pelletized and the supernatant was discarded. The cells were prefixed in 3 % gluteraldehyde for 1 hour and washed in buffer. The cells were then post fixed in 1 % osmium tetraoxide and washed in buffer. After post fixation the cells were blocked with 2 % agarose. The pellet was dehydrated with ascending series of graded alcohol (50% to 100%) and infiltrated by propylene oxide and epoxy resin. The pellet was embedded in siliconised rubber mould with epoxy resin. The pellet was embedded mould was kept in an incubator at 60ºC for 48 hours and then cooled down for sectioning. Ultrathin section (below 100 nm) was made by ultramicrotome (Leica) with diamond knife (Diatome). Ultrathin sections were taken on copper grid and stained with uranyl acetate (double metallic) and Reynold's solution (Sodium citrate and Lead mitrate), which gave the contrast. Sections were observed under electron microscope (Philips 201C, Netherland) and photographed.

Cytomorphological observation

The Cytomorphological details such as diameter of the cell (or breadth- length), nuclear diameter/length, nucleus /cytoplasm ration and granule size were made by using the micrometer. Before measurement, the ocular and the stage micrometer were calibrated. After calibration the measurements were made using the ocular micrometer. The size of the whole cells and the components of cytoplasm was given in µm.

RESULT AND DISCUSSION

Hemocytes were described in the present study on the basis of their structural features, granule size, cell size, shape and the discernible nuclear-cytoplasmic ultrastructural descriptions.

1) Prohemocytes:

Prohemocytes displayed a round/ oval profile with an average diameter of 6.46 µm and were characterized by a high nuclear cytoplasmic ratio. The diameter of the nucleus was measured as 4.37 µm. Small electron dense visicles rough endoplasmic reticulum, cytoskeletal microtubules in the perinuclear area were the discernible structural details of this cell type.

2) Granulocytes:

Granulocytes were characterized by large electron dense, membrane bound granules showing a round or oval profile, with a mean cell length of 11.57 µm and mean cell width of 7.856 µm. Beneath the plasma membrane were seen the microtubules consisting a bundled and ring like structure. The plasma membrane in a few case showed the formation of blunt pseudopodia on its surface. Based upon the size of the granules. Previous investigators on crustaceans classified the granulocytes into small granule (SGH) and large granule (LGH) type. Both cells exhibited a low nucleus to cytoplasm ratio. Besides SGH and LGH, several cells contained a mixture of both smaller and large granulocytes dispersed in cytoplasm as has been reported by Martin *et al.,* (2003) in *Sicynia ingentis*. The differences between the different cell types of granulocytes lie in

the size of the cell, size of the granules, the location of the nucleus and the refractile granules ect. In the present study, all the three type viz., SGH, LGH and mixed granulocytes were observed in the electron microscopic preparations. In the case of LGH, abundant filling the cytoplasm. The SGH Showed dispersed granules with less abundance. Unlike the previous observations the granuocytes in *S. Tranquebarica* showed dumb bell shaped nucleus.

3) Cystocytes:

According to previous studies, they are highly unstable cells and undergo disintegration as soon as the haemolymph is exposed to air or removed from the body of organisms. Hyalinocytes, cystocytes, coagulocytes are terminologically synonymous and are seen in all crustacean forms. However their percentage in THC varies in different forms. These cells in the present study measured 6.65 to 10.45 µm in diameter with a high nuclear/ cytoplasmic ratio (N/C) and with few lysosomes. They were also devoid of granules specifically. In some cells the cytoplasmic streaming on one edge could be noticed clearly. These cells also float passively in plasma. Under phase contrast microscope these cells appear to be refractile in nature. The cystocytes represent the main cell types involved in plasma gelification in forms where their percentage was 60-70 %. Recently martin *et al.,* (2003) have demonstrated that wheat grem agglutinin binds with granulocytes but not with hyaline cells and thereby suggested that this cell type may belong to a distinct cell lineage, in contrast to granulocytes. In *S. Tranquebarica* the identification of coagulocytes was based on the following characteristics. They are round/oval profile, clearly discernible nucleus, absence of granules, low nuclear – cytoplasmic ratio, the streaming nature of cytoplasm and the meagre percentage in THC. Our observations agree with the description made by Ravindranath (1975) on coagulocytes of shore crab, *Emerita asiatica.*

4) Plasmatocyte:

Previous studies on crustaceans revealed this cell type as a rare one among the circulating blood cells (Ravindranath, 1975). In the present study on *S. Tranquebarica* the cells were of variable sizes ranging from 11.4 to 14.25 µm were noticed. Recent electron microscopic studies have characterized the phenotype of these cells with the features such as round or oval profile, circular nucleus, with a central or pericentral nucleolus, less electron dense cytoplasm, round or very oblongated mitochondria and golgi complex, and the presence of more number of highly heterogeneous membrane bound cytoplasmic vesicles (Giulianini, 2003). In the present study, the plasmatocytes were found to be represented rarely in *S. Tranquebarica.* However, they were identified based upon the previous phenotypic characteristics. In addition, these cells showing pseudopodial extensions were clearly discernible. The Plasma membrane also showed occasional investigations in these cells as reported in *Cetonicschema aeruginosa* larvae (Giulianini *et al.,* 2003). The cell cytoplasm also contained granules. Hence, it may be concluded that in *S. Tranquebarica,* besides granulocytes, the plasmatocytes may also be taking part in the phagocytic reactions. Unlike the

previous observations, the plasmatocytes observed in the present study also exhibited the dumb-bell shaped nucleus (Fig 1).

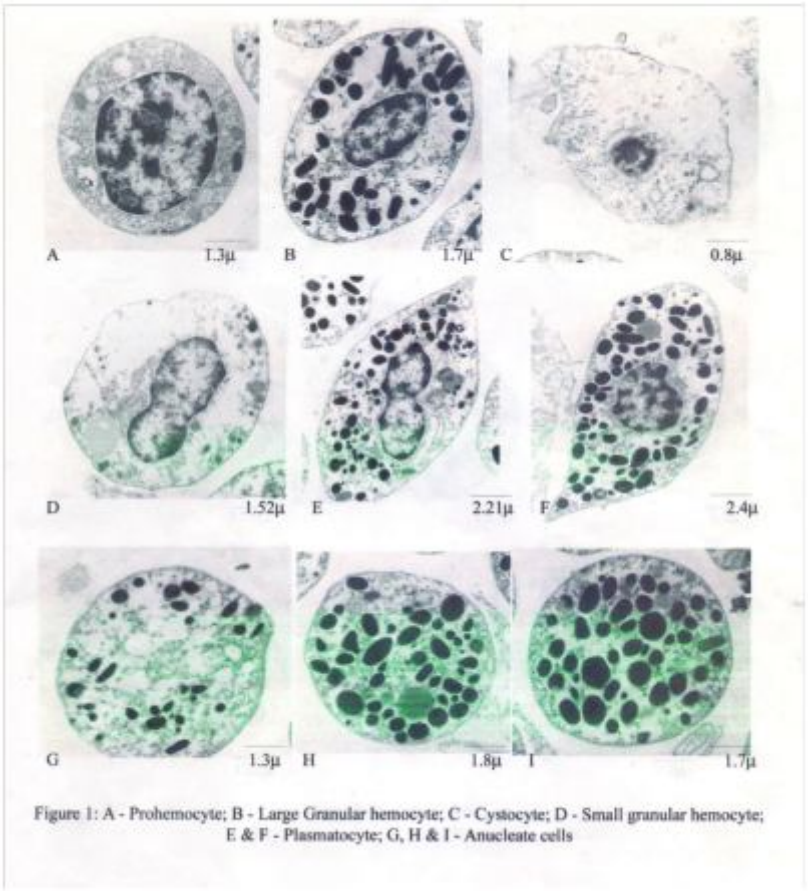

Figure 1: A - Prohemocyte; B - Large Granular hemocyte; C - Cystocyte; D - Small granular hemocyte; E & F - Plasmatocyte; G, H & I - Anucleate cells

5) Podocytes:

Recent studies have reported podocytes in decapods crustaceans. These were identified by their characteristic oblong shape in the present study, the cells measured from 12.5 to 13.3 µm in diameter. Podocytes in crabs were said to possess membrane diaphragms that apparently act to filter the hemolymph. Foreignproteins and smaller viruses were reported to be removed by these podocytes. On the contrary large viruses may be selectively removed via the podocytes localized at the bases of the gill branchial and lamellae. Our observations in *S. Tranquebarica* demonstrated the presence of such oblong cells. But in the gills, such cells were not found as mentioned in *Procambarus clarkia* (Dickson *et al.*, 1991). Probably the gill podocytes could have been shed into the water in rearing tanks. The agile nature of the individuals indicating infection free condition may also be attributed for their absence in gills as they usually abound in gills to nodulate the viral/ bacterial particles.

6) Fixed Phagocytes:

These constitute a category of defensive cells capable of phagocytosis (Ratcliffe *et al.*, 1985). In decapods crabs these were found to be located adjacent to the hepatic arterioles of the hepatopancreas. In size and appearance these resemble the semi granulocytes. Simillar cells were noticed in the sections of hepatopancreas of *S. Tranquebarica* infiltrating in enormous numbers at the base of lobules. As per literature, bacteria and viruses are taken up and phagocytosed by these fixed phagocytes and they may also be destroyed by the ingested bacterial and viral pathogens.

7) Spherule cells:

These cells could be identified by theirlarge size (15 to 22 μ) with refractile granules or spherules. The nucleus is eccentric. The cell size varies and similarly the spherule size varies in diameter. Gregoire, (1955) has described these cells in Limulus polyphemus as "the refractile mulberry like cells" these cells are also seen rarely in decapods. The spherules were reported to represented reserve substances. They are also refractile in apprearance and acidophilic in nature (Ravindranath, 1975). However, these cells were not discernible in the present study.

8) Adipohemocytes:

These are the rare cell type with oval profile and have eccentric and refractile droplets or globules of various sizes (20 to 30 μ). These resemble the small fat body cells of the insects. Ravindranath (1975) has reported that these cells have been observed on rare occasions in the haemolymph of *Emerita asiatica*. However, in the present study, these cells were not seen in *S. Tranquebarica.*

9) Anucleate bodies / cells

Along with the nucleated cell types several cells without a discernible nucleus were observed in both light microscopic and electron microscopic photographs of *S. Tranquebarica* hemocytes. The profile of these cells was more or less uniform with oval or round boundary. The cytoplasm was filled enormously with electron dense granules alongside with a few electron lucent granules or vesicles. Some of the cells were with scant granules. These cells were found to be similar to the progenitor haemoblast cells as seen in the hematopoietic lymphoid tissues of tunicates (Ermak, 1977). He observed the progenitor cells or hemoblasts cells at the centre of lymph nodules with differentiating cells located at the periphery in *Styela clava.* Rowley (1982), in addition, revealed such progenitor cells undergoing differentiation in the blood stream outside the hemotopoietic tissue environment in ascidians. A well defined haemopoietic organs was evident in decapods crustaceans, which lies in close proximity to the ophthalmic artery. These cells which were anucleate probably may represent the progenitor haemoblast cells released from the haemopoietic organ and undergoing the event of differentiation in the blood outside the environment of the haemopoietic tissue. The above cells illustrate and prove the statement of Ratcliffe *et al.*, (1985) that differentiation of cells in crustaceans occurs both in the haemopoietic tissues and the blood. These cells may otherwise also be considered as mitosis undergoing cells in the phase with nucleus not discernible. Even otherwise such anucleate cells with

plenty of granules in their cytoplasm may be released into the blood from the haemotopoietic tissues for specific functions after the dissemination of nuclear information during their formative stage. Such cells also found embedded within the hepatopancreatic lobules, strengthens the above view. However it is premature to conclude about the evidence for such anucleate cells.

According to Martin (Personal Communication), the evidence for such anucleate cells may be derived from light microscopic smear preparations and studies. Light microscopic observations of the smears stained with leishman and Giemsa also revealed the presence of such anucleate cells, in our study. In phase contrast slides, the discernibility of anucleate cells was found to be ambiguous.

Anucleate cells have been observed alongside the population of nucleated cell types. The morphological descriptions of these cells conform to those of granulocytes except the presence of nucleus. The granules of electron translucent and electron dense nature may represent the cytoplasm synthesized materials. Through the possibility of protein synthesis plausible through nuclear DNA has been well established, the fair chance of cytoplasmic DNA contributing to protein synthesis relative to that of nuclear DNA has also been unequivocally established in invertebrate anucleate embryonic cells (Baltus and Brachet, 1962). Similarly the existence of organelle DNA like the mitochondrial DNA has been proved (Piko *et al.,* 1967; Tyler 1967). These extra nuclear DNA supporting protein synthesis has also been verified both in *in vivo* and *in vitro* studies (Giudice, 1960; Nakano and Monroy 1958). Moreover the phenotypic feature of the anucleate cells also drew homology to that of developing and differentiating primary mesenchyme cells in sea urchin embryonic development.

Anucleate cells formation and their existence in the blood circulation may not wholly be unexpected since in the invertebrate development, anucleate eggs development and their divisions through cleavage have been recorded in *Arabacia punctulata* (Harvey, 1935). Likewise cell division without mitotic apparatus in sea urchin eggs has also been witnessed (Holman *et al.,* 2004). Considering these studies which envisioned that anucleate eggs can undergo cleavage and can develop until blastula stage, it may be presumed that such anucleate hemocytes could have originated from the hemotopoietic tissues during proliferation by the unusual orientation of the spindle during divisions of the stem cells and released into the circulation without genetic complement of nucleus. Since circulating cells also undergo mitogenesis and differentiation in crustaceans (Ratcliffe *et al.,* 1985) a similar phenomenon presumably also could have occurred in the haemocoel outside the hematopoietic tissues. Recent study on a species also revealed documented evidence to such blood cell (hemocytes) division through scanning electron microscopy (Silva, 2002). As ionic concentractions of surrounding environment such as the concentrations of calcium and magnesium ions are determinant factors of cells divisions, the possibility of such anucleate cells formation inside the blood may seem to be acceptable (Louis, 1974). Such anucleate eggs have been found in several

invertebrates such as Stentor, triton *Ilyanassa obsolete* and also in frog (Harry, 1970).

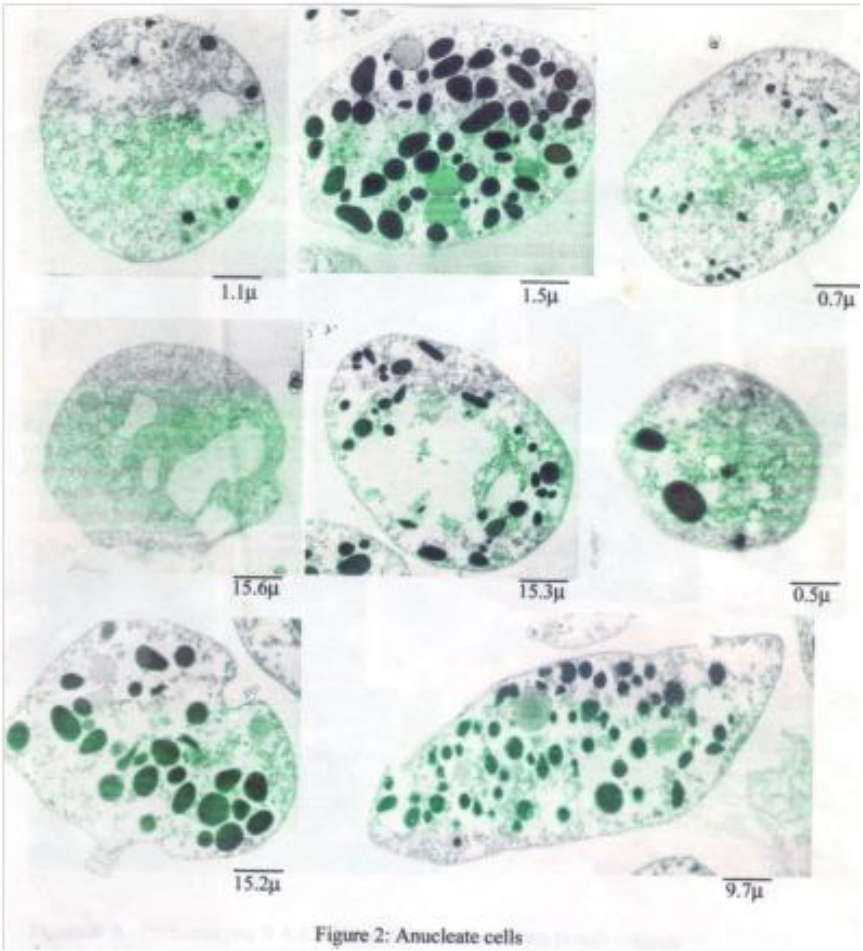

Figure 2: Anucleate cells

It may therefore be suggested that such anucleate cells represent a distinct category with protenaceous granules formed through cytoplasmic DNA directed protein synthesis specifically for some immunological function or they may represent the cells formed fortuitously during mitogensis. Or otherwise they may be suggested to represent the precursor cells without differentiation or in the process of differentiation from anucleate stage to nucleate stage. The statement of Ratcliffe *et al.* (1985) that unlike the vertebrate counterparts, in invertebrates the blood cell differentiation occurs both in the hemotopoietic tissues as well as in blood seems to support the view of ours as a tenable one.

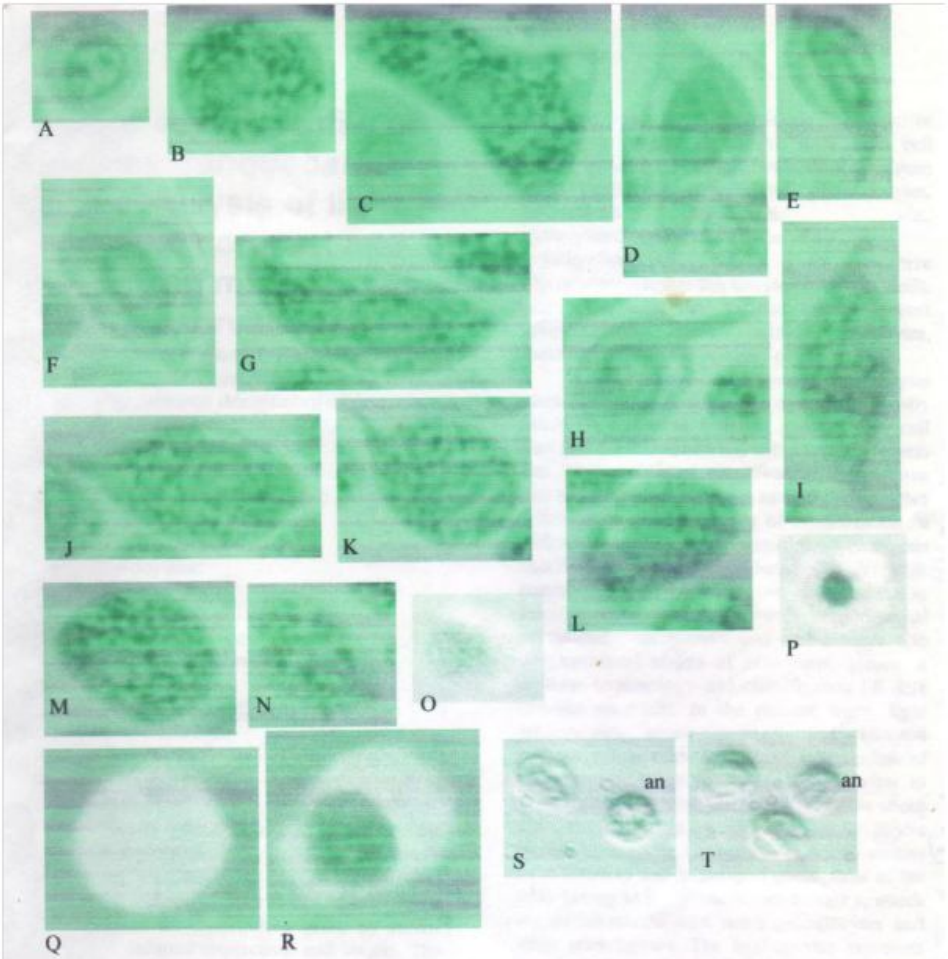

Figure 3: A - Prohemocyte; B & C - Mixed Granular hemocyte (small + large); D, E, F & H - Cystocytes; G - Podocyte; J, K & L - Plasmatocytes; I, M & N - Anucleate cells. O & P - stained with giemsa and leishman, where O - anucelate cell. Q & R - stained with giemsa only, where Q - anucleate cell. S & T - Phase contrast micrographs, showing anucleate cells marked as 'an'.
Scale: A to N - 1 cm = 3.33µ; O & P - 1 cm = 8.99µ; Q & R - 1 cm = 3.93µ;
S & T - 1 cm = 8.62µ. (Figures A to N were obtained from the Method I; O to R from Method II)

Total hemocyte Count:

Total hemocytes count is an indicator of the individual's defence in crustacean species, as the population of circulating hemocytes may indicate whether the host defence system was activated or not (Brehelin *et al.*, 1978) as well as status quo of the host versus pathogen interactions. In this context, their depression in number may be taken to indicate the preponderance of microbial growth and invasion while their increase in number may be construed as the immune potential of the crustacean host against the pathogen. Moreover the

THC represents the characteristic hemotocrit/PCV of a species. In the present study, the mean value of total hemocyte count was found to be 26, 501 cells/µL ±5743.121. The differential percentage of various hemocytes affords the specific role of phagocytic cells and the coagulocytes in microbial infection. A minimum of 150 cells from different stained areas were counted to estimate the differential percentage of cell types. The results revealed 68% of granulocytes, 9.25% cystocytes, 19.45% of Prohemocytes, 1.85% of plasmatocytes and 1.45% of podocytes. The spherule cells, adipohemocytes and oenocytoid cells were not discernible in the present study. The larger number of granulocytes (68%) agrees with the observations made in different decapod species. However, Toh *et al.,* (1991) have revealed that in the Japanese horse shoe crab, *Tachypleus tridentatus*, 99% of the hemocytes belonged to granulocytes exclusively. While granulocytes, plasmatocytes and podocytes were attributed with the phagocytic activity alongside the melanisation and nodulation, the coagulocytes were attributed specifically to bring about the gelification or coagulation and futher prevention of colonization of pathogens. The total hemocytes count (THC) and the differential count in different crustacean forms may not be uniform as species specific variations may be expected. Delineation of such species specific profiles of hemocytes in crustacean would help to understand better their immune strategy.

CONCLUSION

The multifarious cell types such as prohemocytes, small granular hemocytes (SGH), large granular hemocytes (LGH), coagulocytes, podocytes, lamellacytes, oenocytoids, spherule cells, adipohemocytes and their diagnostic characters revealing homology functionally between certain cells such as the phagocytic potential while a specific and distinctive function of plasma gelification exclusively by the cystocytes and the storage of reserve substances in such cells as spherule cells elucidate the oligopotency of hemocytes viz., two or more cell lineage within a tissue forming subsets of variable numbers. The present observation of anucleate cells also adds a new dimension to the hemocytes concept of invertebrate in general and crustaceans in particular. Considering the modern stem cells concept that they undergo both symmetric and asymmetric divisions to produce daughter cells intotwo lineages viz., Somatic line and germ line with equivalent development potency implies that anucleate cells observed in the present study may probably represent the somatic line (Morrison and Kimble, 2006). However it is premature to conclude about the structural and functional status of anucleate cells and it might be worthy of further investigation.

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