Available online at www.ijpab.com

International Journal of Pure & Applied Bioscience

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **3 (3):** 234-240 (2015)

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

ResearchArticle

OPEN ACCESS

Ultrastructure of the hemocytes of Muga Silkworm larva *Antheraea assama* Westwood (Lepidoptera; Saturniidae): a phase-contrast and electron microscopic study

Bhavna Prishnee Baishya*[1], Sunayan Bardoloi[2] and Rupjyoti Bharali[3]
[1]Department of Biotechnology, Gauhati University, Guwahati-781014, Assam, India
[2]Department of Zoology, B. Borooah College, Guwahati-781007, Assam, India
*Corresponding Author E-mail: bhavnabaishya07@gmail.com

ABSTRACT

The ultrastructure of the circulating hemocytes of the fifth instar larvae of Antheraea assama were investigated by means of light microscopy as well as electron microscopy (Scanning Electron Microscopy and Transmission Electron Microscopy). Five types of hemocytes were found in the hemolymph of Antheraea assama and were identified as prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids, in addition to certain preparation artefacts. Plasmatocytes and granulocytes were found to be the most abundant cell types in the hemolymph, whereas oenocytoids the rarest.

Key words*: Hemocytes; Antheraea assama; Prohemocytes; Plasmatocytes; Granulocytes; Spherulocytes; Oenocytoids.*

INTRODUCTION

The hemolymph of insects and other invertebrate groups have cellular inclusions called hemocytes. These hemocytes play important role in the physiology of the organism to which they belong. They are responsible for coagulation of hemolymph[10,11] connective tissue synthesis[23,24,25] wound healing, self recognition, general and specific immune response and opsonisation[14,18,26,27,28]. They form the cellular immune system of many arthropods being involved in phagocytosis and encapsulation[21]. Furthermore, hemocytes produce and store substances which may be discharged after infection such as bacterial substances like lectins and hemolysins and elements of the phenoloxidase system[28]. In some arthropods, hemocytes are also responsible for production and storage of the respiratory pigments[28].

Therefore the study of hemocytes is very important from the physiological point of view and many works have been carried out to ascertain the morphology as well as the physiological and histochemical reactions of hemocytes in different invertebrate groups. But the most important pre-requisite for such studies is a clear classification of different hemocyte types. [lack of which has so far hindered assigning any clear cut physiological function to the possible hemocyte types.] Unfortunately most previous classification of hemocytes has been confusing as it is an accumulation of the works of various workers on various groups of species. As the technique of study and the developmental stages under study varied with different workers, establishment of a uniform classification/terminology became difficult. The result has been the identification of over seventy different hemocyte types. Jones reduced this number to nine. Further advancement in technology, employing autoradiography and electron microscopy limited the number to seven basic types[4,13,16] with certain intermediate types. However, Hagopian[15], Moran[19], etc went a step further and suggested that there may be only one basic type of hemocyte from which all others are derived.

The aim of the present study therefore is the characterisation of the different classes of hemocytes for a reliable uniform classification of the hemocyte types especially in our target species *Antheraea assama* Ww, a sericigenous insect native to the state of Assam, India, and world famous for producing the golden-hued muga silk fibre[6]. The study assumes more importance in the sense that there is scanty data on *A. assama* hemocytes and this is a first attempt of its kind on this species using Phase Contrast Microscope (PCM), Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) technology. According to Arnold[2,3], since a species has distinct hemocyte types, a proper classification of the same can be utilised to clarify the taxonomy of the group.

MATERIALS AND METHODS

Animals: Originally disease free eggs of *A. assama* were obtained from the Nongpoh unit of Central Silk Board, Meghalaya, India. Several generations of this stock have been cultured and maintained in the Sericulture Farm, Khanapara, Assam, India and also in our laboratory for experimental purpose.

Light microscopy (Phase-contrast microscope): Hemolymph drops were obtained from live specimen, i.e. 5th instar, 48hr post-moult larvae, by severing the tip of one of the prolegs, as at this stage full complement of hemocytes are available[7]. Unfixed hemolymph drops were directly collected on clean glass slides, smeared, air-dried and was fixed in methanol following Bardoloi and Hazarika[7]. Hemocytes were then observed under phase contrast optics (make RADICAL, magnification 40X).

Transmission Electron Microscope (TEM): TEM have been performed for identification of the circulating hemocytes of *A. assama* as Chiang *et al.*,[8] reported in their comparative study of light, SEM and TEM of *Blatella germanica* that all hemocytes could be recorded by TEM. Hemolymph samples were obtained from the larvae (5th instar, 48hr post moult) by severing one of the prolegs and collected in microcentrifuge tubes having equal volume of Karnovsky's fixative, washed in 0.1M Sodium Cacodylate buffer (pH:7.2-7.4) and the hemocytes were pelleted at 3000 rpm centrifugation for 30 mins. The pellets obtained were then post-fixed in 1% osmium tetraoxide in the same buffer, serially dehydrated in acetone and embedded via propylene oxide in araldite. Sections were cut on ultramicrotome and collected on 200 mesh copper grids. Sections stained with uranyl and lead citrate for 5 mins each were observed with a TEM [Model: JEM-100X II] [12].

Scanning Electron Microscope (SEM): For observation of hemocytes under SEM, hemolymph samples were collected from 5th instar larvae 48hr post moult as mentioned above. Samples were prefixed in 3% glutaraldehyde in 0.1 M Sodium Cacodylate buffer (pH 7.2-7.4) for 2hrs at 0°C. The specimen were then washed three times (30 mins each) with the same buffer, followed by subsequent post fixation in 1% osmium tetraoxide for 2 hrs, and then washed three times (30 mins each) in distilled water. Following dehydration in a graded series of acetone, they were dried to critical point using CO_2 and mounted on copper stubs with double sided sticky tape and coated with gold palladium. They were then examined under Scanning Electron Microscope [Model: JSM-6360 (Jeol)] at 20kV.

RESULTS

Light and electron microscopic studies have revealed the presence of basically 5 types of hemocytes in *A. assama* hemolymph. These include: Prohemocytes (PRs), Plasmatocytes (PLs), Granulocytes (GRs), Spherulocytes (SPs) and Oenocytoids (OEs).

Light Microscopy (PCM)

Under phase-contrast microscope, plasmatocyes and granulocytes are the most readily distinguishable hemocyte types. Prohemocytes are mostly round or oval and the smallest of the hemocyte types. Plasmatocytes in *A.assama* are easily identifiable because of their spindle-shaped structure. Granulocytes are mostly round and are larger than prohemocytes but smaller than plasmatocytes, and can be identified by the presence of numerous cytoplasmic granules, as observed under phase-contrast microscope. Spherulocytes have characteristic spherule-like protrusions on the cell surface and are generally spherical/elliptical in shape. All other cell types have more or less smooth cell surface. The oenocytoids are the largest of the hemocyte types and also the less frequent cell type in *A. assama*. (Fig. 1)

Electron Microscopy (TEM, SEM)

Prohemocytes: Prohemocytes (size: length=6.164±0.54µm, width=5.18±0.99µm) are the smallest cells of all hemocyte types and are mostly spherical or oval (Fig.2). The nucleus (size: length=3.68±0.24µm, width=2.51±0.38µm) is large as compared to the cell volume with an evident nucleolus and evenly dispersed chromatin. Cytoplasm constitute few cellular organelles and fewer electron dense vesicles.

Plasmatocytes: Plasmatocytes are typically spindle-shaped in *A. assama*, which makes them readily identifiable (Fig.3). These are significantly larger (size: length=11.83±0.31µm, width=4.065±0.78µm) than prohemocytes. The nucleus is the largest (size: length=7.03±0.22µm, width=3.58±0.33µm) among all hemocytes and is mostly elongated or oval with an evident nucleolus. The cytoplasm shows well-developed cell organelles, including small round or very elongated mitochondria, and a number of electron translucent vesicles of variable size. The surface shows few small projections/filopodia.

Granulocytes: Granulocytes (size: length=9.62±0.4µm, width=7.96±0.8µm) show a circular or oval profile and are smaller than plasmatocytes but larger than prohemocytes (Fig.4). The nucleus (size: length=4.28±0.19µm, width=2.41±0.15µm) shows an irregular profile with an evident central nucleolus. Cytoplasm shows small circular mitochondria and numerous vesicles of variable size and density. A characteristic feature of granulocytes is the presence of a large number of electron dense granules (size: diameter=0.8-1.12µm) in the cytoplasm, which makes their identification in TEM/SEM easier. Another consistent feature of granulocytes is the formation of pseudopodia/filopodia on the cell surface.

Spherulocytes: Spherulocytes (size: length=8.53±0.22µm, width=7.15±0.35µm) display characteristic spherule-like (globular) protrusions on the cell surface and are generally spherical to oval in shape (Fig.5). These spherules are formed due to the presence of large electron translucent vacuoles/granules (size: diameter=1.75-2.18µm) in the cytoplasm. Thus, in TEM sections these vacuoles, of variable sizes, tend to obscure the nucleus and as such the nucleus is not prominent in such sections.

Oenocytoids: Oenocytoids are large in size (size: length=8.76±7.2µm, width=5.9±0.2µm), however, they are also rare. Under SEM, oenocytoids appear typically disc-shaped, (Fig.6) and from lateral and dorsal view, these hemocytes are slightly biconcave. However, they generally have smooth cell surfaces without any cytoplasmic projections. Oenocytoids have a large central nucleus (size: length=3.53±0.06µm, width=3.13±0.09µm) and a few, small, electron dense granules in the cytoplasm.

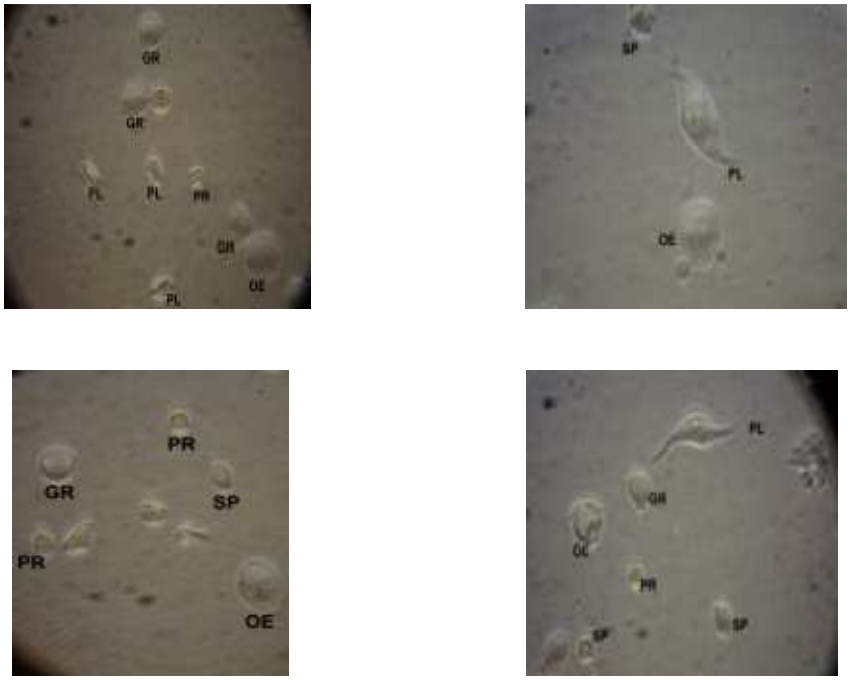

Fig.1: Phase-contrast microscopy images of hemocytes: Prohemocyte (PR), Plasmatocyte (PL), Granulocyte (GR), Spherulocyte (SP) and Oenocytoid (OE)

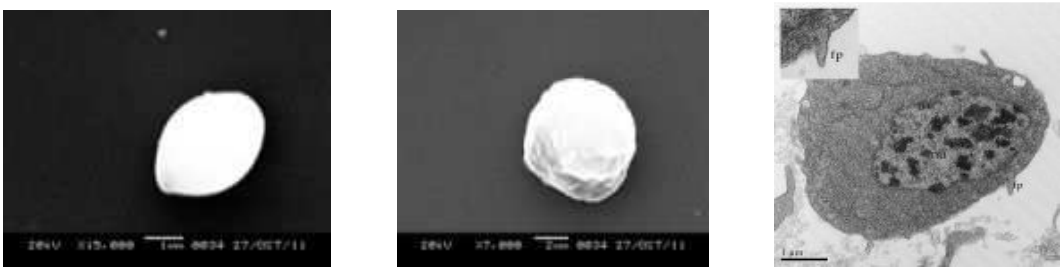

Fig. 2: SEM and TEM ultrastructure of prohemocyte (PR). (From left to right) SEM images of prohemocytes, smallest of the hemocyte types. TEM structure of prohemocyte, showing short filopodia in the inset

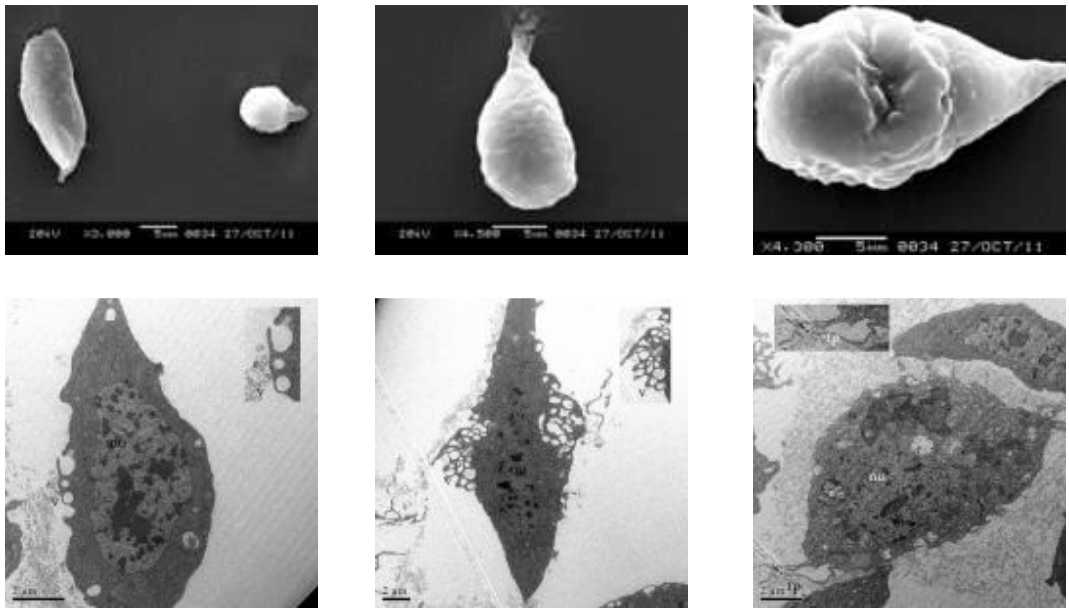

Fig. 3: SEM and TEM ultrastructure of plasmatocyte (PL). (From left to right) Top line: SEM ultrastructure of a plasmatocyte and a smaller granulocyte. SEM image of a plasmatocyte, tapering at one end and giving rise to cytoplasmic processes. SEM image of plasmatocyte, showing a puckered plasma membrane. Bottom line: TEM image of plasmatocyte showing a large nucleus and vesicles (v) along the plasma membrane (inset). Plasmatocyte with network of vesicles (v) (inset). Two different plasmatocytes, one giving rise to long, slender filopodia. (inset).

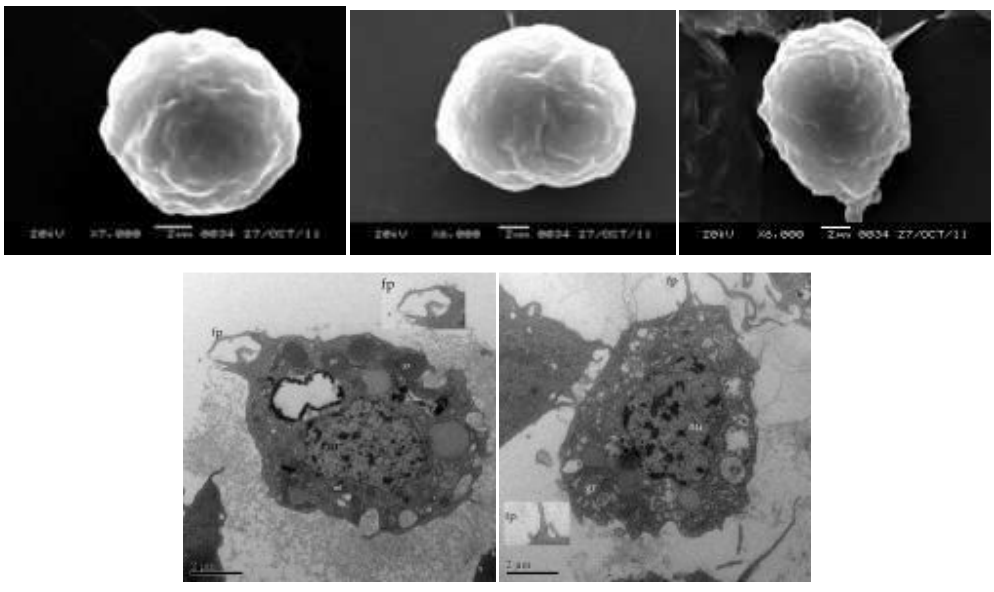

Fig 4: SEM and TEM ultrastructure of granulocyte (GR). Top line: (left to right) SEM ultrastructure of granulocytes, identifiable due to their uneven surface topology, short filopodia and oval or near-spherical shape. Two of the SEM images show granulocytes with fine cytoplasmic processes. Bottom line: TEM images clearly show the electron-dense granules, characteristic of granulocytes, and long, slender filopodia (inset).

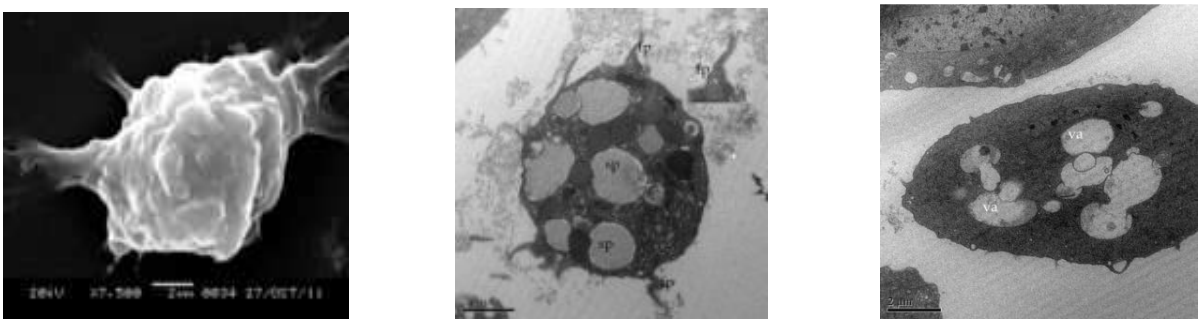

Fig. 5: SEM and TEM ultrastructure of spherulocyte (SP). SEM ultrastructure of spherulocyte showing spherule-like protrusions and several cytoplasmic processes. TEM ultrastructure images showing large spherules (sp)/ vacuoles (va) in the section that obscure the nucleus and several filopodia (inset).

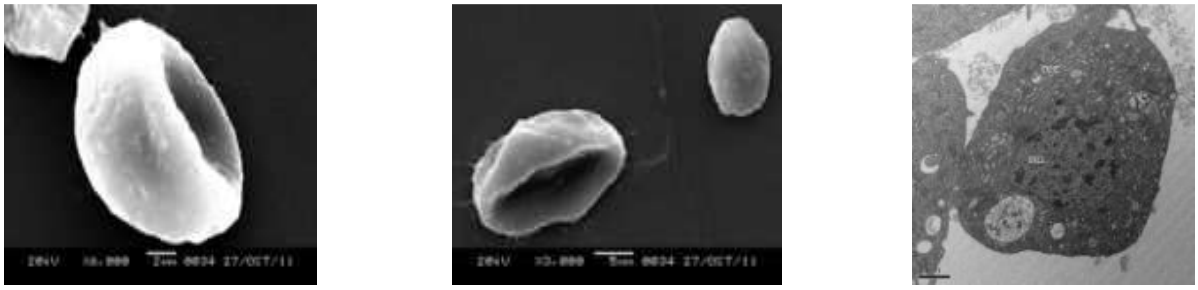

Fig. 6: SEM and TEM ultrastructure of oenocytoid (OE). The two SEM images of oenocytoid show the hemocytes as large, biconcave cells. Second SEM image showing an oenocytoid with a granulocyte. TEM image of oenocytoid showing smooth cell surface with dense cytoplasm.

DISCUSSION

Elimination of variations in the blood picture by comparison of PCM, SEM and TEM results has enabled us to establish the existence of Prohemocyte (PR), Plasmatocyte (PL), Granulocyte (GR), Oenocytoid (OE) and Spherulocyte (SP) as basic hemocyte types in *A. assama.*. Other inclusions observed have to be considered as preparation artefacts. Intermediate cellular forms were also observed which indicate the development of one cell type to another and this would explain the difficulty sometimes encountered in distinguishing cell types. Nevertheless, light and electron microscopic (SEM & TEM) morphology of the hemocytes of *A. assama* was found to correspond to that of other arthropods, particularly the lepidopterans viz. *Bombyx mori*[1], *Agrotis segetum*[5] etc.

Plasmatocytes (PLs) were found to be the most abundant cell types in *A. assama.* The spindle shape of the PLs with cytoplasmic projections, membrane enclosed vesicles are in accordance with the description of other authors in different insect taxa viz *Bombyx mori*[1], *Blatella germanica*[8], *Agrotis segetum*[5], *Pectinophora gossipiella* etc.

Granulocytes (GRs) having characteristic granules of size 0.8-1.12µm are the readily distinguishable type. The granules and the filopodia/other irregular cytoplasmic process conform to the description of GRs in species like *B. Mori*[1], *A. Segetum*[5], *Melolontha melolontha*[9], *Cetonischema aeruginosa*[12] etc.

The spherule cells (SPs) of *A. assama* show distinctive spherule protrusions against the plasmamembrane in SEM which is substantiated by TEM photographs (Fig.5). The presence of membrane bound spherules obscuring the nucleus makes the SPs of *A. assama* easily comparable to those of *B. mori*[1] and *A. Segetum*[5].

The oenocytoids (OEs) of A. assama are the most readily distinguishable type of hemocytes. Being characteristically biconcave with a depression in the centre they are more or less similar to those reported in *B. Mori*[1]. They are also comparable to the ultrastructure of the oenocytoids reported in *Cetonischema aeruginosa* larvae[12].

The structure of the cells named by Akai & Sato[1] as prohemocytes of larval *B. mori*, *Locusta migratoria*[17] and *A. segetum*[5] are very similar in size and shape with *A. assama* both under light microscopy as well as electron microscopy.

Finally it is worth mentioning that in addition to the recognisable hemocyte types, there are some cells, identification of which was very difficult. These might be the intermediate types or have to be considered as preparation artifacts. These kinds of cells were also encountered in electron as well as light microscopic study by Shapiro[22], Xylander[28] etc. to name a few. However, it is not the presence or absence of cell types or developmental stages which is of prime importance, but the role of these various types in the overall physiology of the insect. As such, further study in this regard is most essential.

Acknowledgement: We are grateful to the Central Silk Board, Nongpoh unit, Meghalaya, India for supplying the layings for the experiment. Special thanks to the manager and staff of the Muga Seed Farm, Khanapara, Govt. of Assam, India for allowing us to rear our crops in the farm. We would also like to extend our acknowledgement to the Sophisticated Analytical Instrument Facility (SAIF), instituted at NEHU (North-Eastern Hill University), Shillong, Meghalaya for permitting us to utilise their SEM and TEM facility for this study and also the Institutional Biotech Hub, B. Borooah College for the light microscopy study. This study has been supported by North Eastern Council (NEC), Shillong.

REFERENCES

1. Akai, H. & Sato, S., Surface ultrastucture of the larval hemocytes of the silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) *Int. J. Insect. Morphol. Embryol.* **3(1):** 17-21 (1976)
2. Arnold, J.W., Haemocytology in insect biosystematics: The prospect. *Can. Entemol.* **104:** 655-659 (1972a)
3. Arnold, J.W., A comparative study of the hemocytes (blood cells) of cockroaches (Insecta: Dictyoptera: Blattaria), with a view of their significance in taxonomy. *Can. Entomol*. **104:** 309-348 (1972b)
4. Arnold, J.W. (1974). The hemocytes of insects, pp. 201-254. In *M. Rockstein (ed.).* The Physiology of Insecta, Vol. 5, *2nd ed. Academic Press, New York.*
5. Ayvali, C. & Gul, N., Surface ultrastructure of the larval hemocytes of turnip moth *Agrotis segetum* Denis and Shiff. Lepidoptera:Noctuidae. *Commun. Fac. Sci. Univ. Ank. Serie C.* **6:** 199-204 (1988)
6. Bardoloi, S. & Hazarika, L.K., Seasonal variation of body weight, lipid reserves, blood volumes and haemocyte population of *Antheraea assama. Environ. Entomol.* **21:** 1398-1409 (1992)
7. Bardoloi, S. & Hazarika, L.K., Variation in haemocyte population during different larval instars of *Antheraea assama* and their roles in the defence mechanism of the insect. *J. Assam. Sci. Soc.* **37(2):** 96-102 (1995)
8. Chiang, A.S., Gupta, A.P., Han, S.S., Arthropod immune system I. Comparative light and electron microscopic accounts of immunocytes and other hemocytes of *Blatella germanica* (Dictyoptera: Blattellidae). *J. Morphol.* **198:** 257-267 (1988)
9. Devauchelle, G., Etude ultrastructurale des hemocytes du Coleoptere *Melolontha melolontha* (L.). *J. Ultrastruct. Res*. **34:** 492-516 (1971)
10. Gregoire, C., Blood coagulation in arthropods V. Studies on hemolymph coagulation on 420 species of insects. *Archs. Biol*. **66:** 104-148 (1955)
11. Gregoire, C., Studies by phase-contrast microscopy on distribution patterns of hemolymph coagulation in insects. *Smithson. Misc. Collns.* **134:** 1-35 (1957)
12. Guilianini, P.G., Bertolo, F., Battistella, S., Amirante, G.A., Ultrastructure of the hemocytes of *Cetonischema aeruginosa* larvae (Coleoptera, Scarabaeidae): involvement of both granulocytes and oenocytoids in invivo phagocytosis. *Tiss. and Cell.* **35:** 243-251 (2003)

13. Gupta, A.P. (1979). Hemocytes types: their structures, synonymies, interrelationships and taxonomic significance. In Insect Hemocytes: Development, forms, functions and techniques (Ed. By A.P. Gupta) pp. 85-127. *Cambridge University Press*.
14. Gupta, A.P. (1986). Arthropod immunocytes: Their identification, Structure, Function and Functional Analysis with those of Vertebrate B- and T-lymphocytes. In: Haemocytic and Humoral Immunity in Arthropods, Gupta, A.P. (Ed.). John Wiley and Sons, New York, pp: 3-59.
15. Hagopian, M., Unique structures in the insect granular hemocytes. *J. Ultrastruct.* Res. **36:** 646-658 (1971)
16. Hinks, C.F. & Arnold, J.W., Haemopoiesis in Lepidoptera II. The role of haemopoietic organs. *Can. J. Zool*. **55(10):** 1740-1755 (1977)
17. Hoffmann, J.A., Stoeckel, M.E., Porte, A., Joly, P., Ultrastructure des hemocytes de *Locusta migratoria* (Orthoptere). *C.R. Acad. Sci. (Paris)* **266:** 503-505 (1968)
18. Millar D.A. & Ratcliffe, N.A., The evolution of blood cells: Facts and enigmas. *Endeavour* **13:** 72-77 (1989)
19. Moran, D.T., Fine structure of cockroach blood cells. *Tiss. and Cell*. **3:** 413-422 (1971)
20. Price, C.D. & Ratcliffe, N.A., A reappraisal of insect hemocytes classification by the examination of blood from 15 insect orders. Z. *Zellforsch. Mikrosk. Anat*. **147:** 537-549 (1974)
21. Salt, G. (1970). The cellular defence reactions in insects. Cambridge Monographs in Experimental Biology, No. 16. New York, N.Y.: *Cambridge University Press*.
22. Shapiro, M. (1979). Changes in hemocyte populations. In: Insect Hemocytes (Gupta AP, ed) *Cambridge University Press, Cambridge.*
23. Wigglesworth, V.B., The role of hemocytes in the growth and moulting of an insect- *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol*. **32:** 649-663 (1955)
24. Wigglesworth, V.B., The hemocytes and connective tissue formation in an insect- *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci*. **97**: 89-98 (1956)
25. Wigglesworth, V.B., Haemoocytes and basement membrane formation in *Rhodnius*. J. *Insect. Physiol*. **19:** 831-844 (1973)
26. Xylander W.E.R., Immune defence reactions of Myriapoda- A brief presentation of recent results. In: Thaler K, Meyer E, Schedl W (eds). Advances in Myriapodology (Proceedings of the 8th International Congress of Myriapodology). *Ber. Nat-Med. Verein Innsbruck. Suppl*. **10:** 101-110 (1992)
27. Xylander W.E.R., Immunabwehr bei Gliederfüßern-Wie sich Spinnentiere, Krebse, Insekten und Tausendfüßer gegen Krankheitserreger schützen. Spiegel der Forschung **11:** 27-30 (1994)
28. Xylander W.E.R., Hemocytes in Myriapoda (Arthropoda): a review. *ISJ* **6:** 114-124 (2009)