

Morphological Characterization of Hemocytes in the Adult Linden Bug, *Pyrrhocoris apterus* (L.) (Heteroptera)

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Josef Berger and Klára Slavičková (2008) Morphological characterization of hemocytes in the adult linden bug, *Pyrrhocoris apterus* (L.) (Heteroptera). *Zoological Studies* 47(4): 466-472. The morphology of hemocytes of the adult linden bug, *Pyrrhocoris apterus* (L.) (Heteroptera) was characterized via light (both classical and confocal) and transmission electron microscopy. Based on this characterization, we classified hemocytes into 4 distinct types: prohemocytes, granulocytes, plasmatocytes, and spherulocytes. Differential hemocyte counts are described. We propose using the Pappenheim panoptic stain to identify different hemocyte populations of *P. apterus*. These results are the beginning building blocks of a knowledge base for our ongoing studies of the function of *P. apterus* hemocytes. As certain morphological characteristics are similar to mammalian blood cells which are used in hemotoxicological safety screening, the use of *P. apterus* as a new hematotoxicological biomodel is suggested. <http://zoolstud.sinica.edu.tw/Journals/47.4/466.pdf>

Key words: *Pyrrhocoris apterus*, Hemocyte, Morphology, Cytochemistry.

Circulating hemocytes (sometimes called “blood cells”) play important roles in defense mechanisms against microorganisms in the hemocoel. Cellular defenses refer to hemocyte-mediated responses such as phagocytosis, nodulation, and encapsulation (Schmidt et al. 2001). Several types of hemocytes have been described in insects by their morphological, cytochemical, and functional characteristics. Monoclonal antibodies are used to precisely characterize hemocyte types and also to distinguish different subpopulations of these cells (Mullett et al. 1991, Lavine and Strand 2002).

The most common types of hemocytes are prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipocytes, and oenocytoids. Their characteristics slightly differ in various insect species (Gupta 1979, Ribeiro and Brehélin 2006). As a result, insect hemocytes remain poorly characterized, especially due to a lack of studies in various species. Due to both economic and

ethical problems with the use of vertebrates in biomedical studies, insects have been suggested as alternative biomodels for toxicological preclinical studies (Berger et al. 2003, Gelbič et al. 2006). In addition, insects have been widely used in other fields of biomedical research, such as neuroscience (see Rubinsztein 2002, Bier and Bodmer 2002, Crowther et al. 2004). Hematology is an integral part of preclinical studies on animals (see Berger 1987), and a good knowledge of hemocytes, therefore, is required before insects can reach a level of importance similar to that of vertebrate models in terms of comparative clinical pathology.

Pyrrhocoris apterus is an important biomodel in insect physiology (Socha 1993), but no study has yet been published describing the classification of its hemocytes. As *P. apterus* may also represent an attractive biomodel for comparative preclinical pathology, where hematology is the integral part of an evaluation system, the purpose of this study

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was to describe the morphology of *P. apterus* hemocytes using fundamental hematological procedures.

MATERIAL AND METHODS

We used both adult males and females which were kept at a constant temperature of 26°C under an artificial photoperiod of 16 h of light and 8 h of dark. Animals were fed lime seeds.

The use of other cytological methods than are usually employed for mammals could lead to a classification of different types of blood cells in both vertebrate and invertebrate animals merely

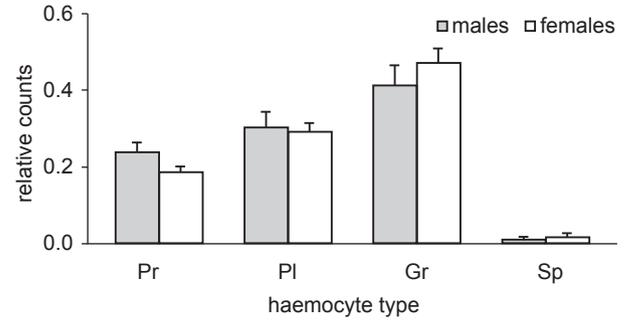


Fig. 1. Differential count of hemocytes in panoptically stained smears. Pr, prohemocytes; Pl, plasmatocytes; Gr, granulocytes; Sp, spherulocytes.

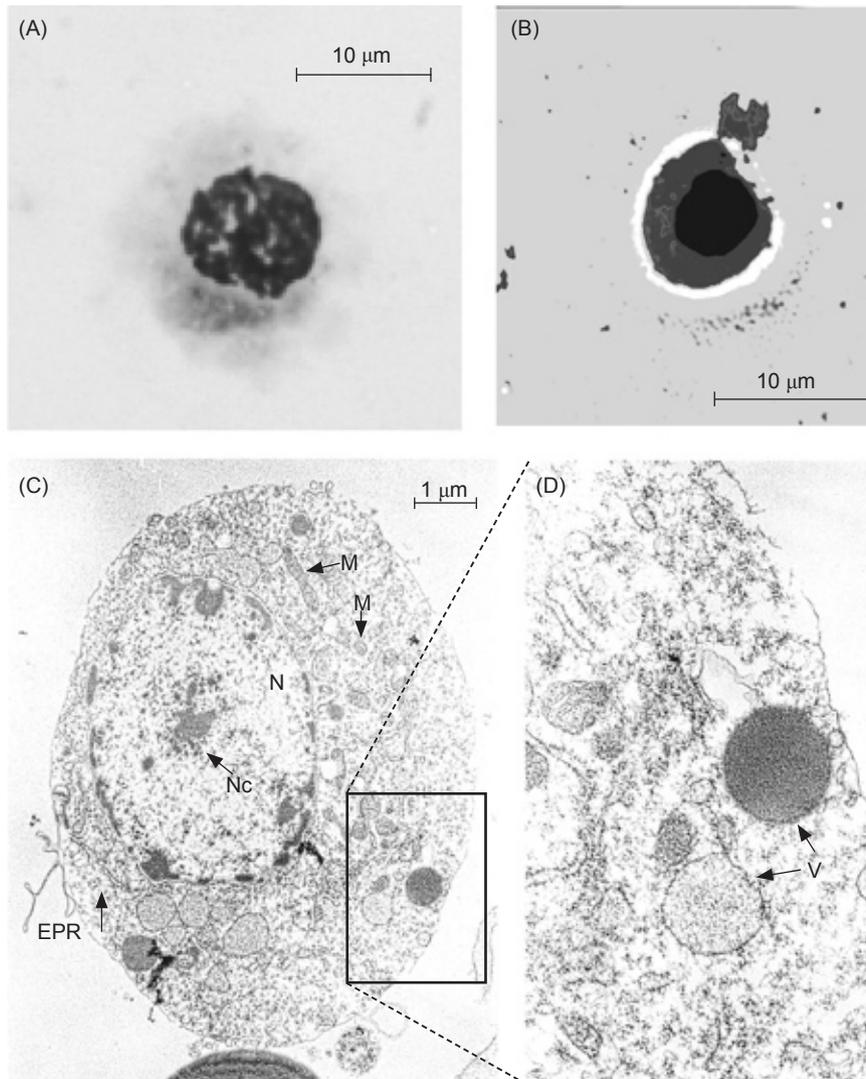


Fig. 2. Prohemocytes. (A) Stained panoptically in the bright field; (B) stained panoptically using laser confocal microscopy; and (C, D) using transmission electron microscopy. N, nucleus; Nc, nucleolus; EPR, endoplasmic reticulum cisternae; M, mitochondrion; V, vesicle.

due to methodological reasons. Therefore, we used light and electron microscopic methods which are fundamental to mammalian hematology to contribute to a search for alternative biomodels among insects. Moreover, we also used laser confocal microscopy to achieve higher resolutions in the framework of light microscopy.

Hemolymph was drawn after cutting off the distal part of an antenna. Smears were immediately made, air-dried, and then stained using the Pappenheim panoptic method. At least 100 hemocytes from each animal were classified to obtain relative counts (so-called differential counts). Laser confocal microscopy was carried out with a LSM 419 inverted microscope (Carl

Zeiss Jena, Germany) at a wavelength of 543 nm (from a HeNe laser), an Apochromat 100/1.4 oil objective, and image analysis software (Carl Zeiss, vers. 3.95).

For transmission electron microscopy (TEM), hemolymph was immediately dispersed in 2.5% glutaraldehyde with 0.2 M phosphate buffer and fixed at 0-4°C for 24 h. Pooled samples from several insects were used. Cells were centrifuged at 440 g for 5 min. OsO₄ (2%) in 0.2 M phosphate buffer for 2 h (the influence of fixation procedures on hemocytes of *P. apterus* was described by Slavíková et al. 2001) was subsequently added before dehydration with acetone (30%-100%). Samples were embedded in EPON resin overnight.

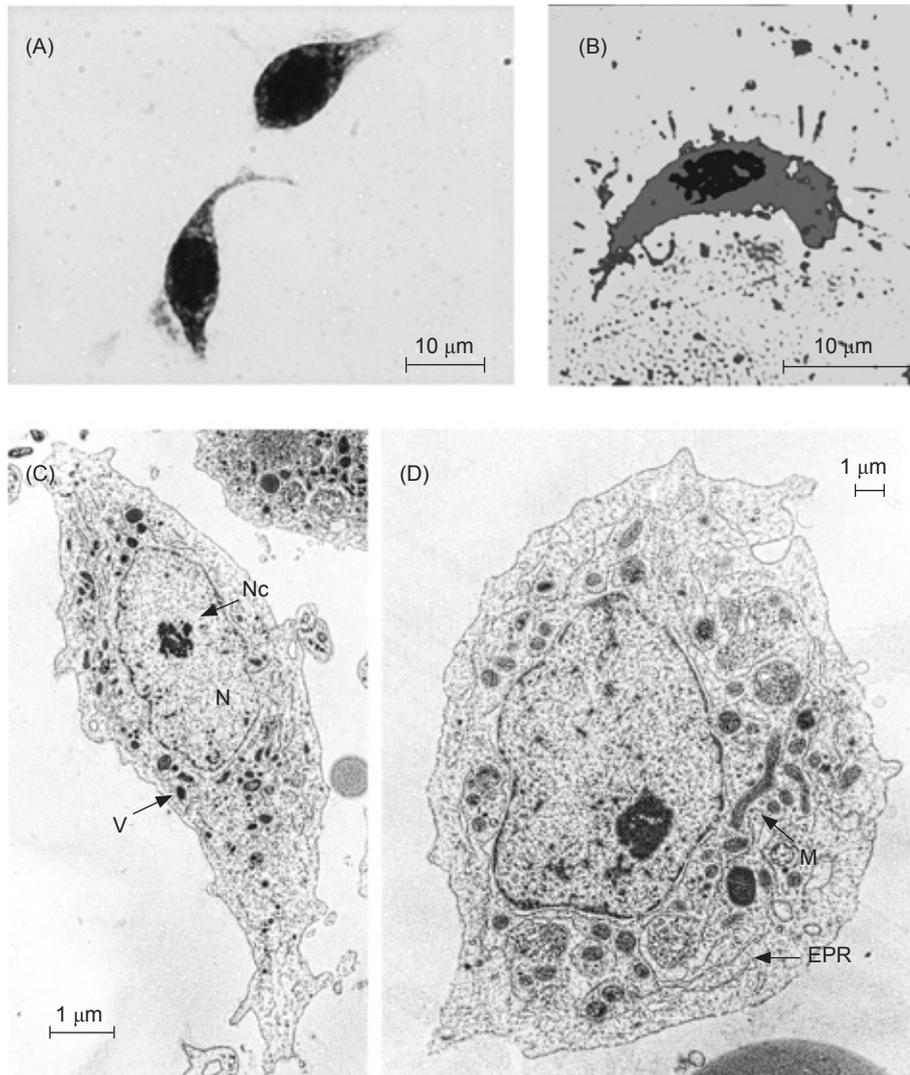


Fig. 3. Plasmatocyte. (A) Stained panoptically using a bright field; (B) stained panoptically using laser confocal microscopy; and (C, D) using transmission electron microscopy. Symbols are as in figure 2.

EPON was polymerized for 48 h at 60°C and then for 24 h at room temperature. Sections were stained for at least 24 h by uranyl acetate in an ethanol atmosphere in the dark for 30 min. JEOL 1010 (USA) and Philips 420 (Netherlands) 80kV microscopes were used.

Resulting data were expressed as the mean \pm SEM from 6-10 animals per group and sex. Results were evaluated using the two-tailed Mann-Whitney *U*-test at a significance level $2\alpha = 0.05$.

RESULTS

We found 4 populations of hemocytes in the hemolymph of *P. apterus*: prohemocytes

(15%-29% of panoptically stained hemocytes on smears), plasmatocytes (23%-40%), granulocytes (34%-60%), and spherulocytes (rare, 0%-4%). No significant differences between differential counts of males and females (Fig. 1) were observed. These 4 hemocyte populations were detected in both panoptically stained smears and specimens for TEM.

Prohemocytes (Fig. 2) are small (3-10 μm), round, oval, or elliptical. Nuclei almost fill the cell, i.e., cells have a high nuclear/cytoplasmic ratio. The cytoplasm is basophilic on optical microscopy, and it contains light granules on electron micrographs. Small amount of endoplasmic reticulum and few mitochondria are scattered in the cytoplasm. Prohemocytes are similar to

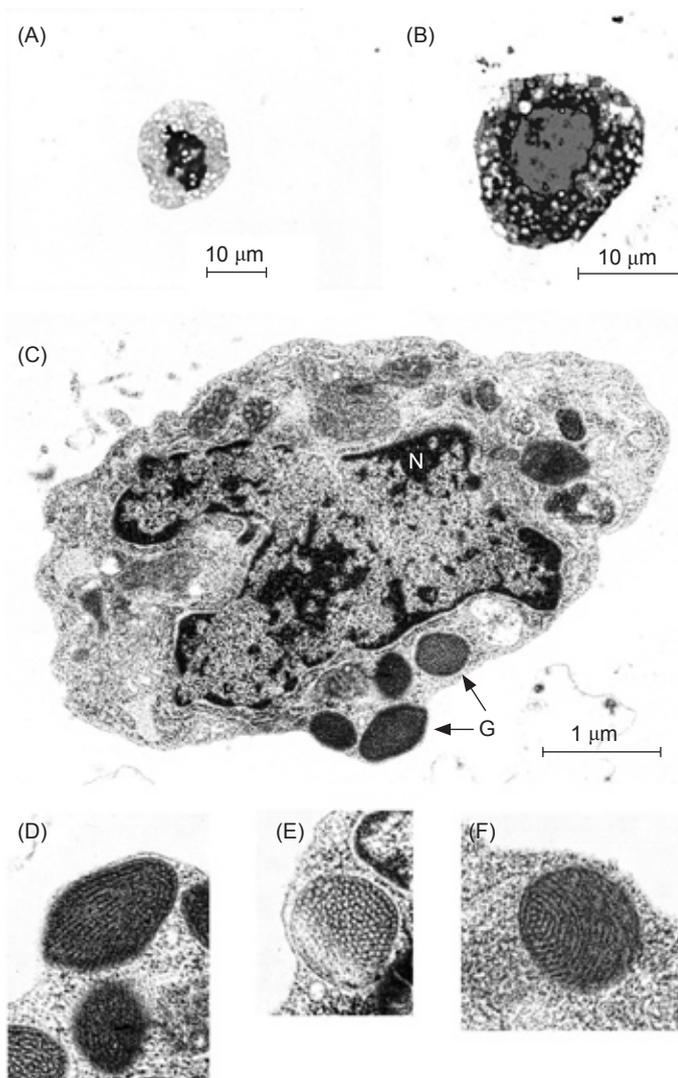


Fig. 4. Granulocyte. (A) Stained panoptically using a bright field; (B) stained panoptically using laser confocal microscopy; and (C, D, E, F) using transmission electron microscopy. G, granule, details of the structured granules are shown in sections D, E, and F; other symbols are as in figure 2.

lymphocytes of vertebrates on panoptically stained smears.

Plasmatocytes (Fig. 3) appear as elongated or spindle-shaped cells. Typical plasmatocytes are 4-25 μm , with a rounded or elongate nucleus, which is 5-11 μm in diameter and contains a massive nucleolus. Electron micrographs show protuberances of the plasma membrane and filopodia. The cytoplasm is basophilic with small granules. There are more endoplasmic reticulum compared with prohemocytes. Sometimes, small vacuoles are present.

Granulocytes (Fig. 4) are the most common type of hemocyte in adult *P. apterus* (Fig. 1). They are small to medium in size (4-20 μm) and elongated, rounded, or spindle-shaped. The nucleus (1-4 μm) is generally located in the cell center and can be lobate. Heterochromatin is much more condensed in panoptically stained granulocytes, and the nucleus in TEM seems to be more electron-dense compared to those of the other cell types. The cytoplasm is slightly basophilic, and contains electron-dense granules (lysosomes). Only a few mitochondria were detected. Numerous cytoskeletal filaments

and cisternae of the endoplasmic reticulum were present. Granulocytes were most easily distinguishable from other hemocytes.

Spherulocytes (Fig. 5) are large (22-48 μm), and rounded or ovoid. They contain large, often eccentric nucleus (12-15 μm in diameter) with nucleoli, surrounded by relatively little cytoplasm and large vesicles. The panoptically stained cytoplasm was basophilic. Large vesicles (up to 11 μm), termed spherules, were often located near the cell surface. We prefer to use the term spherulocyte instead of adipocyte because the vesicles were not characterized by evident sudanophilia (using light microscopy). Panels A and B in figure 5 show morphological variability of these cells by optical microscopy. Cells in panels C and D of figure 5 markedly differ, and can be categorized into different subtypes: C, with electron-dense spherules and D, with electron-transparent spherules.

DISCUSSION

We have characterized the morphology

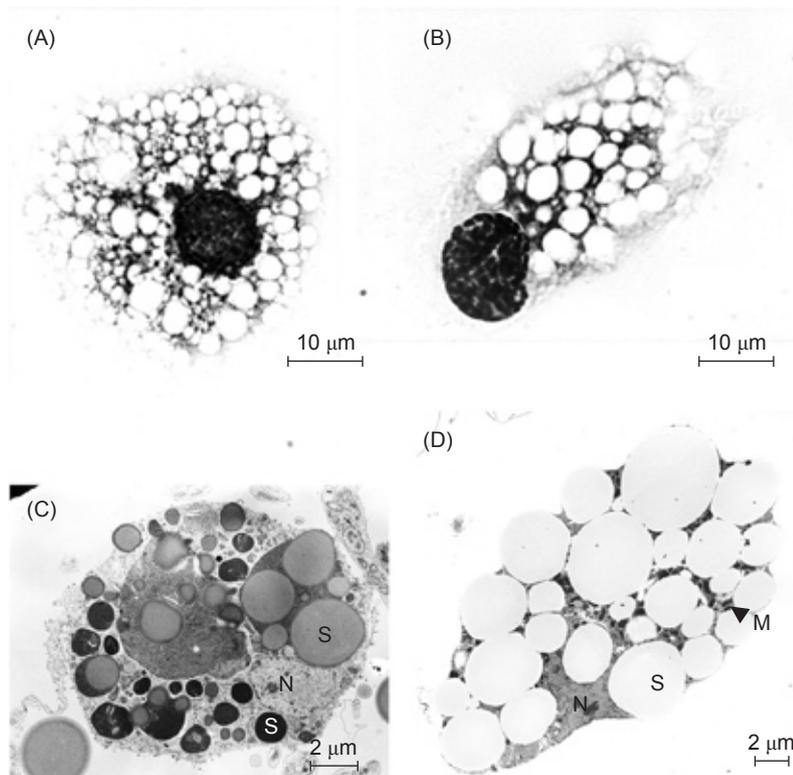


Fig. 5. Spherulocytes. (A, B) Stained panoptically using a bright field; and (C, D) using transmission electron microscopy. S, spherule; other symbols are as in figure 2.

of hemocytes of *P. apterus* using cytochemical methods for light microscopy and TEM. Seven subpopulations of hemocytes were previously found in different insect species, but not all subpopulations are always present in all species (Gupta 1979). Similar to the red cotton bug *Dysdercus koenigii* (Sharma et al. 1998), which also belongs to the Pyrrhocoridae, *P. apterus* has prohemocytes, granulocytes, plasmacytes, and spherulocytes, but has neither adipocytes nor oenocytoids. Spherulocytes, which we observed in *P. apterus*, and adipocytes, described by Sharma et al. (1998) in *D. koenigii*, have similar features in both species. Sharma and co-workers (1998) indicated fat droplets and glycogen rosettes around a fat droplet, but they did not use cytochemical identification which could have confirmed their interpretation of several structures on the electron micrographs. Because of the absence of cytochemistry in the study by Sharma et al. (1998), we cannot eliminate the possibility that similar cells in *D. koenigii* are also spherulocytes.

The light micrograph of an oenocytoid shown in Sharma et al. (1998) resembles a prohemocyte due to the appearance and fine structure of the cytoplasm (defined by Gupta 1979). Moreover, the electron micrograph of the oenocytoid published by Sharma et al. (1998) shows a much higher cytoplasm/nucleus ratio than documented by their published light microphotograph of this type of hemocyte. Thus, it is possible that the above-mentioned oenocytoids in *D. koenigii* are a type of prohemocyte.

The criteria available for comparing differential counts of hemocytes between the 2 genera are not ideal, but no other papers on hemocyte morphology of the Pyrrhocoridae have been published to date. On the other hand, our discussion in the previous 2 paragraphs suggests that the classification of insect hemocytes is not well standardized, which is in stark contrast to the elaborate nomenclature used for vertebrate blood cells. Criteria based only on morphological characteristics, without emphasizing the size of cells as is usual in vertebrate hematology (for example, various populations of circulating lymphocytes do not differ morphologically), could lead to simpler differential counts, with a lower number of hemocyte types.

The morphology of blood cells and their differential count represent fundamental hematological characteristics examined in safety evaluations of newly synthesized compounds

tested on mammals (Berger 1987). We used identical hematological methods as those used in mammals and documented that they can be used to study *P. apterus* hemocytes. These cells are similar to vertebrate leukocytes. This is in contrast to erythrocytes and platelets, which are not present in insects due to the existence of different mechanisms for oxygen transport and coagulation. Vertebrate leukocytes have immunological functions similar to those of insect hemocytes. The morphology and differential counts of mammalian leukocytes provide both fundamental and relatively cheap data, and they are, therefore, frequently examined in both human and veterinary laboratory medicine.

It is interesting that our results show that *P. apterus* prohemocytes are similar to human lymphocytes, plasmacytes are similar to human monocytes, and granulocytes are similar to human neutrophils. The phylogenetic distance between *P. apterus* and humans indicates that hemocytes can only be used for hemotoxicological screening, and not in chronic preclinical studies, but this screening would be much cheaper and without ethical or legal limitations (see Berger 2005) compared to vertebrate biomodels. In terms of the many millions of dollars spent on laboratory rodents for pharmacological screening in the world every year, research on insect hemocytes, as demonstrated in this paper, could offer novel entomological and economic benefits.

To summarize, the present results characterize 4 population of hemocytes in *P. apterus* using techniques which are fundamental to both human and veterinary hematology.

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