Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 82(3):431-438, jul./set. 1987

THE HEMOCYTES OF *PANSTRONGYLUS MEGISTUS* (HEMIPTERA: REDUVIIDAE)

MARGHERITA ANNA BARRACCO*, ROZEMARY DE OLIVEIRA* & BRUNO SCHLEMPER JR**

Universidade Federal de Santa Catarina, *Departamento de Biologia **Departamento de Microbiologia e Parasitologia, Caixa Postal 476, 88049 Florianópolis, SC, Brasil

Five hemocyte types were identified in the hemolymph of Panstrongylus megistus *by phase contrast and common light microscopy using some histochemical methods. These are:* Prohemocytes, *small cells presenting a great nucleus/cytoplasm ratio;* Plasmatocytes, *the most numerous hemocytes, are polymorphic cells mainly characterized by a large amount of lysosomes;* Granulocytes, *hemocytes very similar to plasmatocytes which contain cytoplasmic granules and are especially rich in polysaccharides;* Oenocytoids, *cells presenting a small nucleus and a thick cytoplasm; they show many small round vacuoles when observed in Giemsa smears and many cytoplasmic granules under phase microscopy;* Adipohemocytes, *very large hemocytes, presenting many fat droplet inclusions which could correspond to free fat bodies which entered the hemolymph. Only prohemocytes and plasmatocytes can be clearly classified; all the other hemocyte types have a more ambiguous classification.*

Key words: hemocyte – *Panstrogylus megistus* – Hemiptera

As far as hemipteran hemocytes are concerned, the main studies were carried out on *Rhodnius prolixus*. Wigglesworth was the first to describe the hemocytes of this reduviid and to observe their involvement with ecdysis (1933, 1955, 1979), wound healing (1937), connective tissue (1956) and basement membrane (1973) formation. Jones (1965) also described the hemocytes of *R. prolixus*, using the phase contrast microscopy, though with some disagreement with Wigglesworth's classification. He also analysed (1967) the variation of hemocyte number in relation to ecdysis and feeding.

Lai-Fook (1968, 1970) studied the involvement of the hemocytes of the same reduviid in wound repair and described these cells ultrastructurally, basing her classification on Jones and Wigglesworth descriptions.

Very few other hemipteran species were studied for this purpose. Among them, we mention the work of Dorn (1979) with *Oncopeltus fasciatus* and Ali & Ilyas (1985) with *Dysdercus cingulatus*.

In Brazil, the reduviid *Panstrongylus megistus* is one of the most important vectors of the protozoan *Trypanosoma cruzi*, which causes Chagas disease. In the present study we identify and classify the circulating hemocytes of *P. megistus* by phase contrast and common light microscopy, using also some histochemical methods.

This work was supported by a research grant from CNPq.

Received February 3, 1987.
Accepted April 14, 1987.

MATERIAL AND METHODS

All hemocyte analyses were performed upon sylvatic fourth and fifth instar and adult *Panstrongylus megistus* from the island of Santa Catarina, not infected by *T. cruzi*. These animals were reared and maintained in our laboratory under controlled temperature (25°–28°C) and humidity (60–80%) and were fed with chicken blood once every two weeks. The life cycle of sylvatic *P. megistus* of the island of Santa Catarina is circa one year in our laboratory. The fourth instar period lasts about 85 days and the fifth instar nymphs take about a hundred days to attain the last moult and reach the adult stage (Steindel et al., in press).

To collect the hemolymph, the insects were maintained at 4°C for 10 minutes for immobilization. The hemolymph was always obtained from a severed leg or antenna.

Hemocyte identification and classification

Phase contrast observation – Fresh, unfixed, unstained, coverslipped samples of hemolymph, producing thin films, were observed under the phase contrast microscope. These wet smears highly facilitated hemocyte identification.

Giemsa stained smears – Hemolymph smears were air dried, fixed with methanol for 10 min and stained with Giemsa diluted with phosphate buffer (1:3), pH=7.0 from 20 to 30 min (Barracco & Cestari, 1984).

Other staining methods were also tried (May-Grünwald Giemsa, hematoxylin-eosin), but Giemsa gave the best results.

Gomori method for acid phosphatase (Lysosomes) – Hemolymph smears were fixed in 10% formol calcium, pH=7.0 for 15–20 min, rinsed with running water and incubated with Gomori substrate for 30 min at 37°C (Pearse, 1968). Next, the smears were rinsed with distilled water, immersed in a weak (1%) aqueous solution of ammonium sulfide for 1–2 min, washed again with running water and mounted. In control experiments, smears were incubated without sodium β-glycerophosphate.

Sudan Black B for lipids – Hemolymph smears were fixed in 10% Baker formol-calcium, pH=7.0 for 15-20 min, rinsed with running water, treated with 50% ethanol for 3 min and immersed in a Sudan Black B solution in 70% ethanol (Pearse, 1968). Slides were then, immersed in 50% ethanol, washed with distilled water and glycerin mounted.

Periodic acid + Schiff (PAS) for Carbohydrates – Hemolymph smears were fixed in 10% Baker formol-calcium, pH=7.0 for 30 min, submitted to 1% periodic acid, washed with distilled water and submitted to Schiff reagent for 30 min. After that time, the smears were washed for 3 min in each of consecutive sulfurous water bathes, left in running water for about 30 min and mounted (Pearse, 1968).

To remove glycogen, some hemolymph smears were submitted to 0.5% amylase in 0.004M acetate buffer, pH=5.5 for 3h at 37°C before being treated with periodic acid (Pearse, 1968).

Alcian Blue, pH=1.0 for Sulfated Glycosaminoglycans and sulfomucins – Hemolymph smears were fixed in 10% Baker formol-calcium, pH=7.0 for 30 min, washed with running water for 2h and submitted to 1% Alcian Blue in 0.1N hydrochloric acid, pH=1.0 for 18h at room temperature. After this time, the smears were dehydrated in two changes of absolute ethanol, cleared in xylene and mounted (Ashhurst, 1979).

Alcian Blue pH=2.6 for Sialic Acid residues on Glycoproteins and Carboxyls of Glycosaminoglycans – Hemolymph smears were fixed and washed in the same way as for Alcian Blue pH=1.0 and stained with 1% Alcian Blue in 3% acetic acid, pH=2.6 for 18h at room temperature. Afterwards, the smears were washed for 5 min with distilled water, dehydrated rapidly, cleared with xylene and mounted (Ashhurst, 1979).

RESULTS

By comparing the results obtained by the different methods employed, we were able to distinguish five hemocyte types in *P. megistus* hemolymph:

Prohemocytes – Small (8-11μm) and spherical cells (Figs. 1A, 2A and 2B). The nucleus (6-9μm) generally fills the cytoplasm almost entirely. The thin cytoplasm layer shows marked basophilia for Giemsa staining. They practically do not react to the histochemical tests applied (Table I). At times, they can be observed undergoing mitotic division (Fig. 2B).

Plasmatocytes – These are the most abundat hemocytes circulating in the hemolymph. They are larger (10-30μm) than prohemocytes (Figs. 1B, 1C, 2C to G) and, when observed on wet smears by phase contrast, they show many thin pseudopods, looking as polymorphic amoebocytes. The nucleus (6-15μm) is, in general, centrally located. In Giemsa smears the cytoplasm is less basophilic than prohemocytes and oenocytoids. At times, it shows some vacuoles (Fig. 2C). These cells are especially reactive to the Gomori method for acid phosphatase (Table I), presenting many dark granules which correspond to lysosomes. Such abundance of lysosomes may indicate their involvement in phagocytosis which we actually found (Figs 2F and 2G). At times, they are seen phagocytizing oenocytoids (Fig. 2G). Some plasmatocytes are reactive to the Schiff (Table I) and may contain sulfated glycosaminoglycans and sulfomucins as indicated by the Alcian Blue pH=1.0 staining. They are also seen undergoing mitosis (Fig. 2E).

Granulocytes – Hemocytes very similar in size (10-30μm) and shape to pasmatocytes when observed in Giemsa smears (Figs. 1D and 3A). By phase contrast microscopy, which is a much better method for their identification, they usually show scattered cytoplasmic granules and when they present pseudopods they are, in general, thicker and shorter than in plasmatocytes (Fig. 1D).

Oenocytoids – Round, oval or elongated (9-28μm) hemocytes (Figs. 1E, 1G, 3B and 3D) showing a marked surface stability and never presenting pseudopods, even after a long time of observation by phase microscopy. Their nucleus is usually smaller (5-10μm) than those of other hemocyte types and can be eccentrically located. Sometimes, it shows characteristic chromatin condensed masses (Fig. 3C). The cytoplasm is thick and homogeneous and clearly basophilic in Giemsa staining. They usually present typically small spherical vacuoles (Figs. 3B and 3D) and sometimes scattered cytoplasmic inclusions. In phase contrast microscopy, they show many cytoplasmic granules instead of the small vacuoles (Figs 1E to G). Oenocytoids are practically negative to the Gomori method

and slightly reactive to the other histochemical tests (Table I). They are the second most numerous hemocytes.

TABLE I

Histochemical Reactions

Histochemical tests	PR	PL	GR	OE	AD
Gomori method	0-1	2-4	0-2	0	0
Sudan Black B	0	0-1	0-2	0	3-4
PAS	0	0-3	1-4	0-2	3-4*
Alcian Blue pH = 1.0	0	0-2	1-3	0-1	0-1
Alcian Blue pH = 2.6	0	0-1	1-2	0-1	0-1

Histochemical reactions or stainings applied to 4th and 5th instars and adults of *Panstrongylus megistus*. The blood cells are represented by PR (prohemocytes); PL (plasmatocytes); GR (granulocytes); OE (oenocytoids) and AD (adipohemocytes). Reaction intensity was scored as follows: 0 – negative reaction or staining; 1 – weak; 2 – moderate; 3 – strong; 4 – very strong.

*The fat droplets of the adipohemocytes are not reactive to the PAS but the surrounding cytoplasm is strongly positive. After the amylase digestion, we could conclude that the cytoplasm of the adipohemocytes is very rich in glycogen.

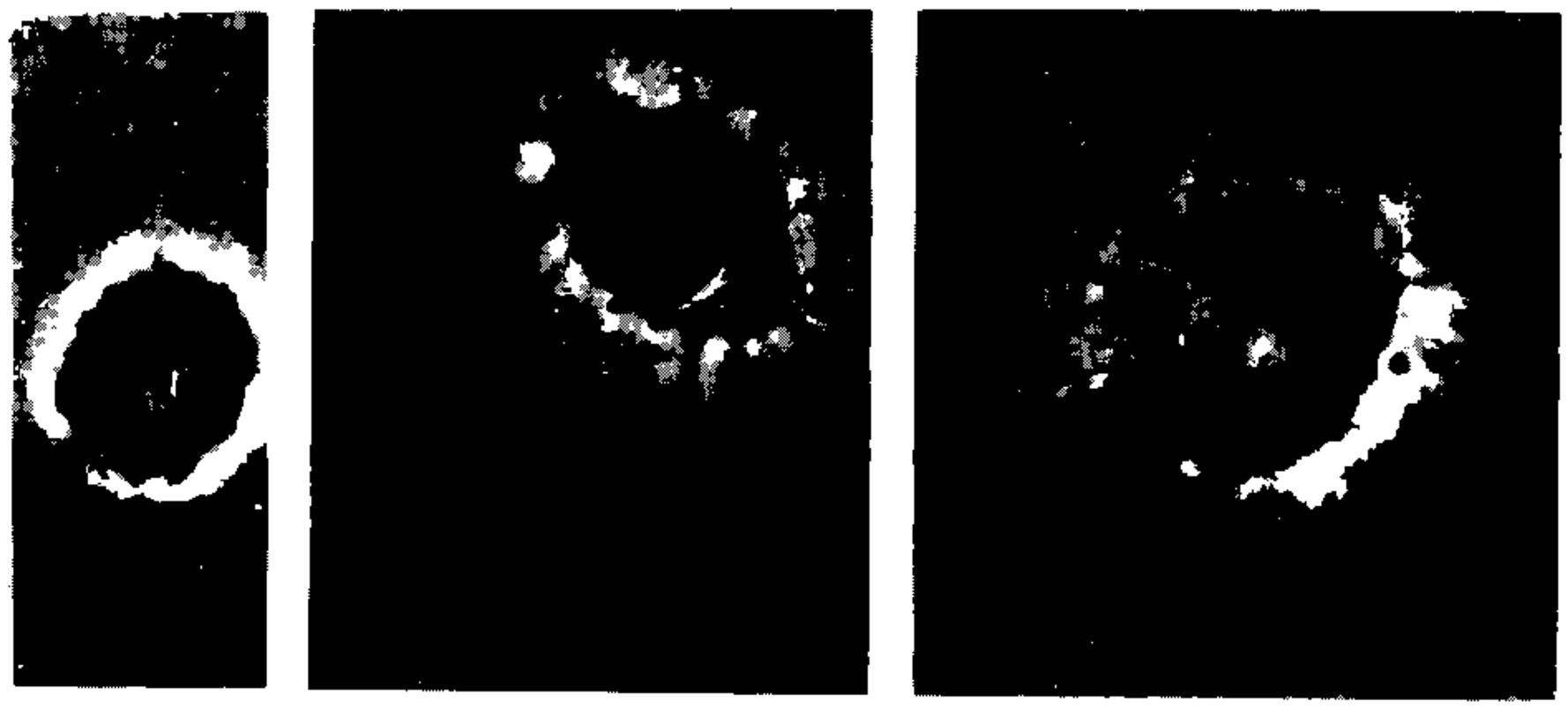
Adipohemocytes – These are usually very large hemocytes (30-90μm) and look like circulating fat bodies (Figs. 1H, 3E and 3F). Their small nuclei (6-9μm) are in general eccentric and the cytoplasm presents many brilliant fat-like droplets when observed under phase microscopy. In Giemsa staining, the fat droplets are removed by the methanol fixation and we could see many vacuoles of different sizes in their place (Figs. 3E and 3F). Adipohemocytes are extremely reactive to Sudan Black B (Table I), which confirms the presence of a large amount of lipids. The fat droplets are negative to PAS but the surrounding cytoplasm strongly reacts to the Schiff reactive. This reveals a high glycogen content which is confirmed by amylase digestion (Table I). Adipohemocytes are not always found in the hemolymph of *P. megistus*. They increase in number near the ecdysis and are very scarce in the adults. When they are present in large numbers, the hemolymph takes a characteristically turbid and yellowish appearance.

DISCUSSION

Insect hemocytes classification is still controversial because these cells present a great morpho-physiological variability and are also very susceptible to the methods employed in their study. Moreover, there is a lack of uniformity in the terminology used by the authors for their classification. This makes an effective comparison of the hemocytes of the different insect orders even more dificult.

In *R. prolixus*, for example, Wigglesworth (1933, 1955, 1956) described four hemocyte types which he called: proleukocytes, oenocytoids, amoebocytes and lipocytes. In 1973, however, the same author reclassified as plasmatocytes and adipocytes the two latter cell types according to a terminology more frequently used. He also observed in this reduviid, two other less frequent cell types that he called large granular and agranular hemocytes. On the other hand, Jones (1965), using phase contrast microscopy, described five different hemocyte types in *R. prolixus* which he termed: prohemocytes, plasmatocytes, granulocytes, oenocytoids and adipohemocytes. He disagreed with Wigglesworth's classification, particularly in the case of oenocytoids and large agranular cells that Jones classified as granulocytes and oenocytoids, respectively. He pointed out the use of phase microscopy for hemocyte classification and considered it to be one of the most useful methods for this purpose. Also, in the same reduviid, Lai-Fook (1970) described five hemocyte types based on Jones'(1965) classification, with the exception of Jones' granulocytes that she called "granulocytophagous cells". Apparently, Lai-Fook could have misunderstood the term "granulocytophagous" introduced by Jones in *R. prolixus* to characterize unmistakable plasmatocytes which were seen, at times, engulfing granulocytes. Lai-Fook considered to be granulocytophagous cells, the fusiform hemocytes presenting many cytoplasmic granules. Her granulocytophagous cells seem to correspond to the granulocytes described by Jones in the same reduviid. Since Lai-Fook's granulocytophagous are not plasmatocytes and are never seen phagocytizing granulocytes, we do not see any reason why they should be classified in this way.

Among other hemipteran species, Dorn (1979) described the hemocytes of *Oncopeltus fasciatus* based on the classification of Lai-Fook (1970), while Ali & Ilyas (1985) studied the hemocytes of *Dysdercus cingulatus* among which they found spherulocytes and cystocytes which are not usually referred to exist in other hemipterans.



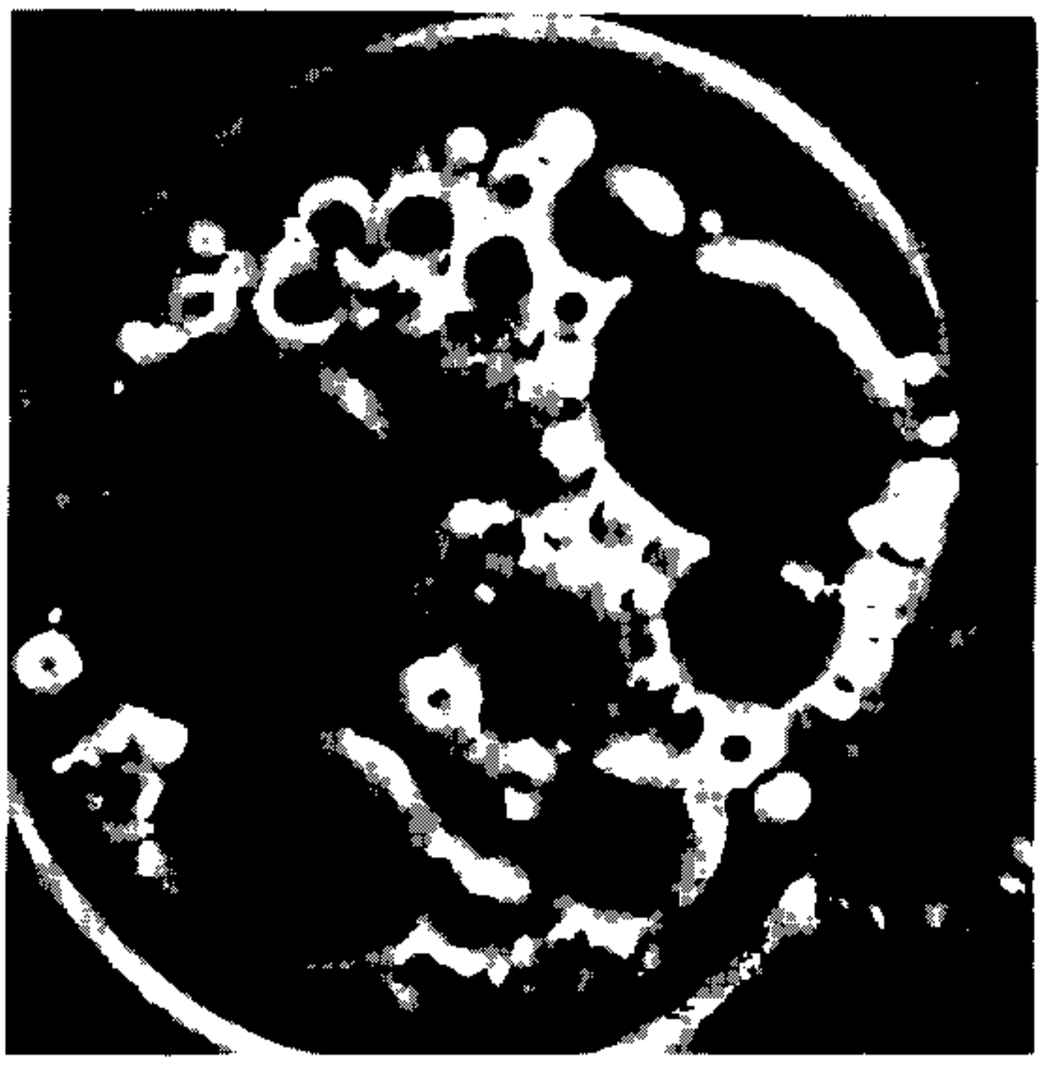


Fig. 1: Phase contrast micrographs. A: Prohemocyte. B and C: Plasmatocytes showing thin filamentous pseudopods. D: Granulocyte. E-G: Oenocytoids. H: Adipohemocyte. Bar = 5μm.

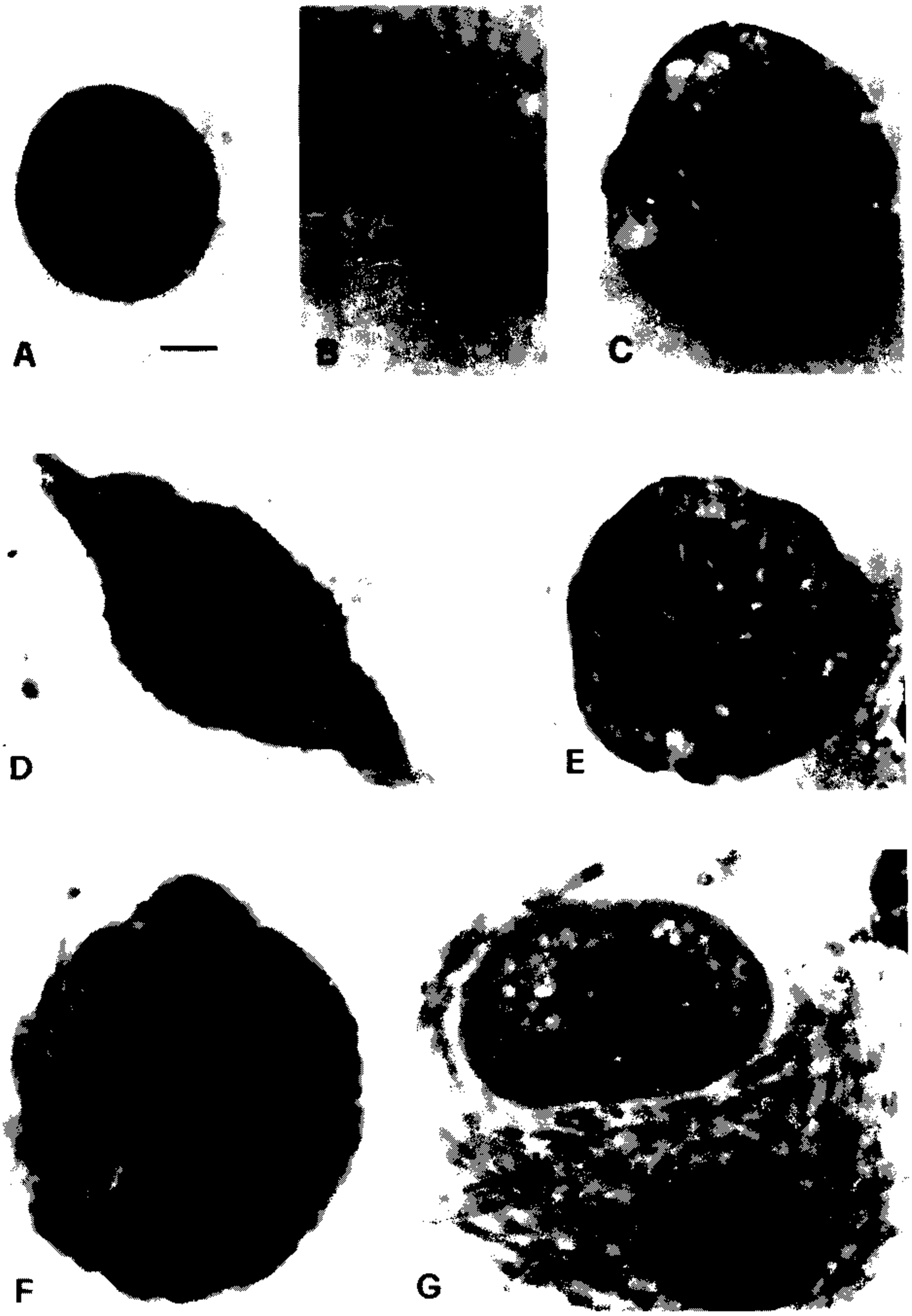


Fig. 2: Hemolymph smears stained by Giemsa. A and B: Prohemocytes; in B telophase. C-G: Plasmatocytes; in E metaphase; in F phagocytosis of two chicken erythrocytes (arrowheads); in G phagocytosis of several bacteriae (asterisks) and an oenocytoid (arrow). Bar = 2μm.

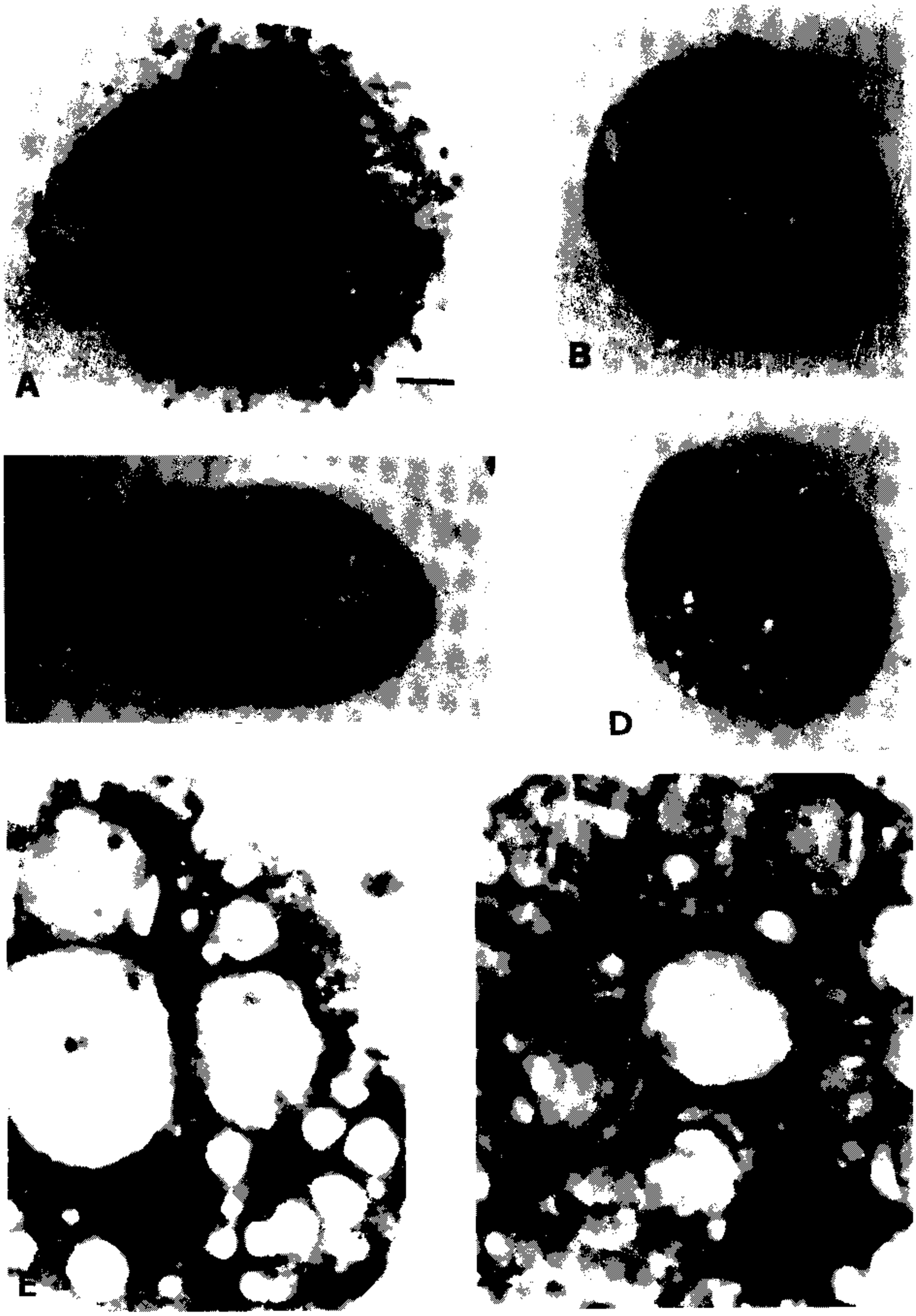


Fig. 3: Hemolymph smears stained by Giemsa. A: Granulocyte. B-D: Oenocytoids, several shapes. E and F: Adipohemocytes. Bar = 2μm.

Concerning the hemocytes of *P. megistus* we can conclude that its prohemocytes and plasmatocytes certainly correspond to the proleukocytes-prohemocytes and plasmatocytes already described in *R. prolixus* as well as in other insect species (see review by Gupta, 1979). We also found plasmatocytes engulfing what we considered to be oenocytoids (Fig. 2G) according to Wigglesworth's classification which, on the other hand, should correspond to the granulocytes described by Jones. Thus, these phagocytizing plasmatocytes should be the granulocytophagous of *R. prolixus*. We preferred to call oenocytoid the "endocyted" cell, because its morphological characteristics are very similar to the oenocytoids of other insect species (see reviews by Gupta, 1979, and Rowley & Ratcliffe, 1981). The oenocytoids are usually described as presenting a very stable surface, showing no pseudopods or filopods when observed by phase microscopy; their nucleus is usually small and eccentrically located; their cytoplasm is particularly thick and homogeneous, containing sometimes several kinds of polymorphic inclusions. For these reasons, we believe that this hemocyte type found in *P. megistus* must correspond to the oenocytoids described in other insects. On the other hand, our so-called oenocytoids in *P. megistus* show several granules when observed by phase microscopy (Fig. 1E to G) and are thus, similar to the granulocytes described by Jones (1965) in *R. prolixus*. In agreement with Jones, we also observed that the oenocytoids of *P. megistus* are much more numerous than in other insect species (see review by Shapiro, 1979). Thus, we actually believe that the classification of this hemocyte type is still controversial and denominations such as granulocytes and cystocytes could eventually be used.

The granulocytes of *P. megistus* are very similar to plasmatocytes (Fig. 3A) and they can easily be confused, especially when the granulocytes present only few granules when stained by Giemsa. Studies of wet smears by phase microscopy are very useful for their identification (Fig. 1D), since granules can be more easily seen and also because they present more conspicuous and short pseudopods instead of the thin thread-like expansions observed in plasmatocytes (Fig. 1D). Granulocytes are reactive to the Schiff (Table I) which indicates the presence of carbohydrates as in *R. prolixus* (Wigglesworth, 1956, 1973). Their staining by Alcian Blue (Table I) is probably due to the presence of sulfated glycosaminoglycans or sulfomucins. Plasmatocytes may also contain the same carbohydrates as in granulocytes but are particularly reactive to the Gomori substrate (Table I) which reveals the presence of a large amount of lysosomes. Thus, the plasmatocytes must be involved in such defense mechanisms as endocytosis, as we actually found (Figs. 2F and 2G).

Adipohemocytes are also very controversial cells. In *R. prolixus*, Jones (1965) and Wigglesworth (1973) believed that they were not true hemocytes and that they actually should correspond to free fat bodies which entered the hemolymph. The adipohemocytes of *R. prolixus* and *P. megistus* strongly differ from the adipohemocytes described in Lepidoptera (Shrivastava & Richards, 1965; Beaulaton, 1968) which originate from plasmatocytes and contain mainly mucopolysaccharides. The adipohemocytes of *P. megistus* contain, as mentioned before, a large amount of lipids droplets (Table I). The cytoplasm surrounding the fat inclusions is very rich in glycogen since it is very reactive to the Schiff, but fail to react with it after amylase digestion (Table I). Adipohemocytes are the unique hemocytes which are not always present in the hemolymph of *P. megistus*, although they seem to be more frequent than in *R. prolixus* (Wigglesworth, 1933, 1973; Jones, 1965). Owing to their morpho-cytochemical features the adipohemocytes of *P. megistus* could effectively correspond to free fat bodies that enter the hemolymph as it is supposed to happen in *R. prolixus*.

We can thus conclude that, in *P. megistus*, only the prohemocytes and plasmatocytes can be clearly classified by phase contrast and common light microscopy using some histochemical methods. All other hemocyte types have a more ambiguous classification.

RESUMO

Os hemócitos de *Panstrongylus megistus* (Hemiptera: Reduviidae) – Cinco tipos de hemócitos foram identificados na hemolinfa de *Panstrongylus megistus* através da microscopia de contraste de fase e de luz, usando alguns testes histoquímicos: *Pró-hemócitos*-células pequenas que mostram uma grande relação núcleo-citoplasmática; *Plasmatócitos*-células polimórficas, que se caracterizam principalmente pela sua grande abundância em lisossomos – são os hemócitos mais numerosos; *Granulócitos*-células muito semelhantes aos plasmatócitos que contêm grânulos citoplasmáticos particularmente ricos em polissacarídeos; *Enocitóides*-hemócitos que apresentam um núcleo pequeno e cujo citoplasma basofílico revela-se muito denso e homogêneo – mostram uma série de pequenos vacúolos esféricos quando observados nos esfregaços corados pelo Giemsa, mas a microscopia de fase revela uma grande quantidade de pequenos grânulos ao invés de vacúolos; *Adipohemócitos*-hemócitos muito grandes que con-

têm uma grande quantidade de inclusões lipídicas – poderiam corresponder a células do corpo gorduroso que, destacando-se, penetram na hemolinfa. Somente os pró-hemócitos e os plasmatócitos podem ser classificados com precisão; os outros tipos podem gerar ambiguidade de classificação.

Palavras-chave: hemócitos – *Panstrongylus megistus* – Hemiptera

ACKNOWLEDGEMENTS

The authors would like to express sincerest thanks to M. Steindel for his contributions to this research. Thanks are also due to Dr. G. Amirante, University of Trieste, for critical reading of the manuscript.

REFERENCES

ALI, F. & ILYAS, M., 1985. The hemocytes of *Dysdercus cingulatus* (Pyrrhocoridae: Hemiptera). *Biologia* (Lahore)., *31* :115-128.

ASHHURST, D.E., 1979. Histochemical methods for hemocytes, p. 581-599. In: A.P. Gupta, *Insect hemocytes.* Cambridge, University Press, Cambridge.

BARRACCO, M.A. & CESTARI, A.N., 1984. Studies on the hemocytes of *Trichosia pubescens* (Diptera: Sciaridae) larvae. *Brazil. J. Genetics., VII* (3) :451-475.

BEAULATON, J., 1968. Étude ultrastructurale et cytochimique des glandes prothoraciques du vers à soie aux quatrième et cinquième âges larvaires. La tunica propria et ses relations avec les fibres conjonctives et les hemocytes. *J. Ultrastruct. Res., 23* :474-498.

DORN, A., 1979. Ultrastructure of differentiating hemocytes in the embryo of *Oncopeltus fasciatus* (Insecta: Heteroptera). *Cell Tissue Res., 187* (3) :479-488.

GUPTA, A.P., 1979. Structure and taxonomic significance, p. 85-128. In: A.P. Gupta, *Insect hemocytes.* Cambridge University Press, Cambridge.

JONES, J.C., 1965. The hemocytes of *Rhodnius prolixus. Biol. Bull. Woods Hole., 129* :282-294.

JONES, J.C., 1967. Normal differential counts of hemocytes in relation to ecdysis and feeding in *Rhodnius. J. Insect Physiol., 13* :1133-1141.

LAI-FOOK, J., 1968. The fine structure of wound repair in an insect *(Rhodnius prolixus). J. Morphol., 124* :37-78.

LAI-FOOK, J., 1970. Haemocytes in the repair of wounds in an insect *(Rhodnius prolixus). J. Morphol., 130* :297-314.

PEARSE, A.G.E., 1968. *Histochemistry, Theoretical and Applied.* Vol. I and II, 3rd. Churchill, London.

ROWLEY, A.F. & RATCLIFFE, N.A., 1981. Insects, p. 421-488. In: N.A. Ratcliffe & A.F. Rowley, *Invertebrate Blood Cells.* Vol. II. Academic Press, London.

SHAPIRO, M., 1979. Changes in hemocyte populations, p. 475-523. In: A.P. Gupta, *Insect hemocytes.* Cambridge University Press, Cambridge.

SHRIVASTAVA, S.C. & RICHARDS, A.G., 1965. An autoradiographic study of the relation between hemocytes and connective tissue in the wax moth, *Galleria mellonella. Biol. Bull. (Woods Hole)., 128* :337-345.

WIGGLESWORTH, V.B., 1933. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae: Hemiptera), with special reference to the function of the oenocytes and of the dermal glands. *Q. J. Microsc. Sci., 76* :269-318.

WIGGLESWORTH, V.B., 1937. Wound healing in an insect, *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol., 14* :364-381.

WIGGLESWORTH, V.B., 1955. The role of the haemocytes in the growth and moulting of an insect, *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol., 32* :649-663.

WIGGLESWORTH, V.B., 1956. The haemocytes and connective tissue formation in an insect, *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci., 97* :89-98.

WIGGLESWORTH, V.B., 1973. Haemocytes and basement membrane formation in *Rhodnius. J. Insect Physiol., 19* :831-844.

WIGGLESWORTH, V.B., 1979. Hemocytes and growth in insects, p. 303-318. In: A.P. Gupta, *Insect Hemocytes.* Cambridge University Press, Cambridge.