THE HEMOCYTES OF PANSTRONGYLUS MEGISTUS (HEMIPTERA: REDUVIIDAE)

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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 – Panstrongylus megistus – Hemiptera

As far as hemipteran hemocytes are concerned, the main studies were carried out on Rhodnius prolixus. Wiglesworth was the first to describe the hemocytes of this reduvid 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 Wiglesworth'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 reduvid in wound repair and described these cells ultrastructurally, basing her classification on Jones and Wiglesworth 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 Dytiscus cingulatus.

In Brazil, the reduvid 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.

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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 40°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.

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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 baths, left in running water for about 30 min and mounted (Pearse, 1968).

To remove glycoprotein, 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 abundant 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 stainers 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 plasmatocytes 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 chromat condensates 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**

<table>
<thead>
<tr>
<th>Histochemical Tests</th>
<th>PR</th>
<th>PL</th>
<th>GR</th>
<th>OE</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2-4</td>
<td>0-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sudan Black B</td>
<td>0</td>
<td>0-1</td>
<td>0-2</td>
<td>0</td>
<td>3-4*</td>
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<tr>
<td>PAS</td>
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<td>0-3</td>
<td>1-4</td>
<td>0-2</td>
<td>3-4*</td>
</tr>
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</tr>
</tbody>
</table>

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 (oecocytoids) 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 glycojen.

**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 difficult.

In *R. prolixus*, for example, Wigglesworth (1933, 1955, 1956) described four hemocyte types which he called: proleukocytes, oecocytoids, 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, oecocytoids and adipohemocytes. He disagreed with Wigglesworth’s classification, particularly in the case of oecocytoids and large agranular cells that Jones classified as granulocytes and oecocytoids, 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. 2: Hemolymph smears stained by Giemsa. A and B: Prohemocytes; in B telophase. C-G: Plasmocytes; in E metaphase; in F phagocytosis of two chicken erythrocytes (arrowheads); in G phagocytosis of several bacteria (asterisks) and an oenocytoid (arrow). Bar = 2μm.
Fig. 3: Hemolymph smears stained by Giemsa. A: Granulocyte. B-D: Oenocytes, several shapes. E and F: Adipohemocytes. Bar = 2µm.
Concerning the hemocytes of *P. megistus* we can conclude that its prohemocytes and plasmacytocytes certainly correspond to the proleukocytes-prohemocytes and plasmacytocytes already described in *R. prolixus* as well as in other insect species (see review by Gupta, 1979). We also found plasmacytocytes 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 plasmacytocytes 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 cytocyctes could eventually be used.

The granulocytes of *P. megistus* are very similar to plasmacytocytes (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 plasmacytocytes (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. Plasmacytocytes 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 plasmacytocytes 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 plasmacytocytes 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 plasmacytocytes 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 nucleo-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; *Enocitoides*-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; Adipo-hemócitos-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 plasmaticócitos podem ser classificados com precisão; os outros tipos podem gerar ambiguous.

Palavras-chave: hemócitos – Panstrongylus megistus – Hemiptera

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REFERENCES


