

Morphological Characterization of the Leukocytes in Circulating Blood of the Turtle (*Phrynops hilarii*)

Caracterización Morfológica de Leucocitos Circulantes en la Sangre de la Tortuga (*Phrynops hilarii*)

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PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O. Morphological characterization of the leukocytes in circulating blood of the turtle (*Phrynops hilarii*). *Int. J. Morphol.*, 25(4):677-682, 2007.

SUMMARY: The *Phrynops hilarii* specie of turtle has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by Leishman and analyzed under light and transmission electron microscope. It was not observe any cellular type with similar characteristics to neutrophils in mammalian group. We believed, based on the data obtained in this study that the heterophils have a morphofuncional analogy with another neutrophils belonged to mammalian group. This conclusion is being supported in many recent studies found in the literature.

KEY WORDS: Turtle; Morphology; Leukocytes; Blood.

INTRODUCTION

In mammals, leukocytes are easily identified on the basis of their morphological differences and the enzymes and other proteins stored in their cytoplasmic granules (Bainton & Farquhar, 1968, 1970; Bainton *et al.*, 1971; Weller, 1991; Dvorak *et al.*, 1991, 1994). Neutrophils are highly specialized phagocytic cells involved in ingestion, death, and degradation of invading microorganisms (MacCall *et al.*, 1971; Roos *et al.*, 1983; Bainton, 1988). Eosinophils are cells that actively participate in the defense against parasitic infections, in the regulation of hypersensitivity reactions, and in the destruction of cancer cells (Kay, 1985; Dvorak *et al.*, 1991; Weller).

Studies have been conducted on lower vertebrates in order to understand biological roles of leukocytes in defense mechanisms, and to establish phylogenetic studies and new experimental models. Some investigators have demonstrated the existence of 2 forms of eosinophils in the blood of turtles, one of them a mature form and the other an immature form (Jordan & Flippin, 1943; Charipper & Davis, 1932), whereas others have stated that there are 2 distinct cell lineages, i.e. neutrophils and eosinophils (Ryerson, 1943; Taylor *et al.*, 1963; Wood & Ebanks, 1984). Because of the wide morphological variation of these cells in different animal

species, it is impossible to characterize them solely on the basis of morphology.

Veterinary haematology has relied on classical Romanowsky staining (e.g. Leishman, Wright and Giemsa) to identify erythrocytes, thrombocytes and leukocytes, but cellular classification of these leukocytes is not always reliable using classical staining methods. Neutrophils are present in some animals, but this cell types have been reported in a few species (Barber & Westermann, 1978; Tavares-Dias & Moraes). Immature leukocytes also can be present in circulating blood (Meseguer *et al.*, 1994; Tavares-Dias & Moraes, 2004). Thus, cytochemical staining of piscine leukocytes may be particularly useful for identification of cellular lineage and may suggest cell function.

Apart from being useful for identifying cell types in blood and tissues, cytochemical staining is also critical for identifying immunological cell types associated with developmental and pathological processes (Burrows & Fletcher, 1987; Meseguer *et al.*; Lorenzi, 1999; Ueda *et al.*, 2001; Petrie-Hanson & Peterman, 2005). Presence of glycogen (Veiga *et al.*, 2000; Ueda *et al.*; Vale *et al.*, 2002; Rough *et al.*, 2005) and alkaline phosphatase (Meseguer *et*

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al.; Burrows *et al.*, 2001) in leukocytes may be associated with phagocytosis. This requires the consumption of energy from both endogenous and exogenous sources (Hayhoe & Quaglino, 1994; Ueda *et al.*). Peroxidase is a lysosomal enzyme, which takes part in intracellular digestion and modulation of phagocytic activity of leukocytes (Hayhoe & Quaglino; Veiga *et al.*; Ueda *et al.*; Vale *et al.*; Azevedo & Lunardi, 2003). Esterases are enzymes also related to cellular defense, facilitating diapedesis, cell migration through tissue, toxic product and microorganism inactivation and tumour cell destruction (Hayhoe & Quaglino; Casaletti-Rosa & Lunardi, 1997; Azevedo & Lunardi). The need to identify these features in leukocytes encouraged numerous studies on turtles (Zinkl *et al.*, 1991; Burrows *et al.*; Ueda *et al.*; Tavares-Dias & Moraes; Palic *et al.*, 2005; Petrie-Hanson & Peterman).

By the fact that the *Phrynops hilarii* specie has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by Leishman and analyzed under light and transmission electron microscope.

MATERIAL AND METHOD

All the aspects of this research were approved by local ethics committee. It was used in this study six *Phrynops hilarii* turtles, obtained at Guaíba river, Porto Alegre, Rio Grande do Sul, Brazil. The animals were lodged for one week at Central Animals House, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The blood of these animals was removed by needle aspiration performed on lateral vessels of the neck. The histological process and staining of this blood was performed by Leishmann stain. These histological images were obtained at a photomicroscope using the Leica IM 50 program connected to Leica DMLB2 microscope.

The collected blood was done using heparinized beakers, centrifuged by 15 minutes at 1000rpm at room temperature. The blood plasma was eliminated and the leukocyte suspension was processed by electron microscopy analysis. Samples of the leukocyte suspension were fixed by Karnowsky solution- glutaraldehyde at 2%, paraphormaldehyde at 2% and cacodilat solution at 0.1M pH 7.4, with 0.05% of calcium chlorite, during 2 hours at room temperature. The blood samples were post-fixed in osmium tetroxide at 1% and cacodilat solution 0.1M, during two hours at the same temperature, and later, observed by CM-100 - Philips transmission electron microscope.

RESULTS

It was found in the present study six types of leukocytes in the turtle blood, *Phrynops hilarii* specie, basophiles, eosinophils, lymphocytes, monocytes, neutrophils and thrombocytes. Basophiles presented spherical conformation with segmented nucleus, spherical granules in cytoplasm (Fig. 1A). Eosinophils were defined as spherical shape and peripheral nucleus, with cytoplasm filled by oval granules (Fig. 1B). Small and spherical lymphocytes, with eccentric nucleus, were observed in almost all cytoplasm (Fig. 1C). Monocytes cells were found in circulating blood, characterized by oval and peripheral nucleus and abundant cytoplasm (Fig. 1D). Neutrophils were found after light microscopy analysis, showing a spherical nucleus and heterophilic aspect, this cellular type was found in electron microscopy analysis, presenting segmented and heterophilic nucleus, and cytoplasm with elongated granules (Fig. 1E). Thrombocytes showed elliptic conformation, with a little cytoplasm and nucleus with elliptic shape too (Fig. 1F).

DISCUSSION

By the fact that the *Phrynops hilarii* specie has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by Leishman and analyzed under light and transmission electron microscope.

Studies related to leukocytes characterization in turtles do not present a consensus in relation to the description of these leukocytes. Research studies related to ground turtles belonged to the species *Terrapene carolina* and *Gopherus polyphenus*, identified in blood samples the following cellular types, basophiles, eosinophils, lymphocytes, monocytes, neutrophils and thrombocytes (Ryerson, 1943). However, in 1962, Head & Rogers working with four species of turtles did not related in their conclusion, the presence of basophiles in blood samples. In a similar study, performed in blood tissue of the turtle *Chelonia mydas*, it was not observed the presence of monocytes and it was considered the heterophils cells as neutrophils (Wood & Ebanks, 1984). In 2004, Munoz & Fuente, studying lymphoid tissues belonged to *Mauremys caspica* specie of turtle, found heterophils cells after the resultant histological analysis. Studies involving another species aiming to study the leukocytes under light microscopy analysis, showed in the turtles species *Padocnemis expansa* and *Emys orbicularis*, the presence

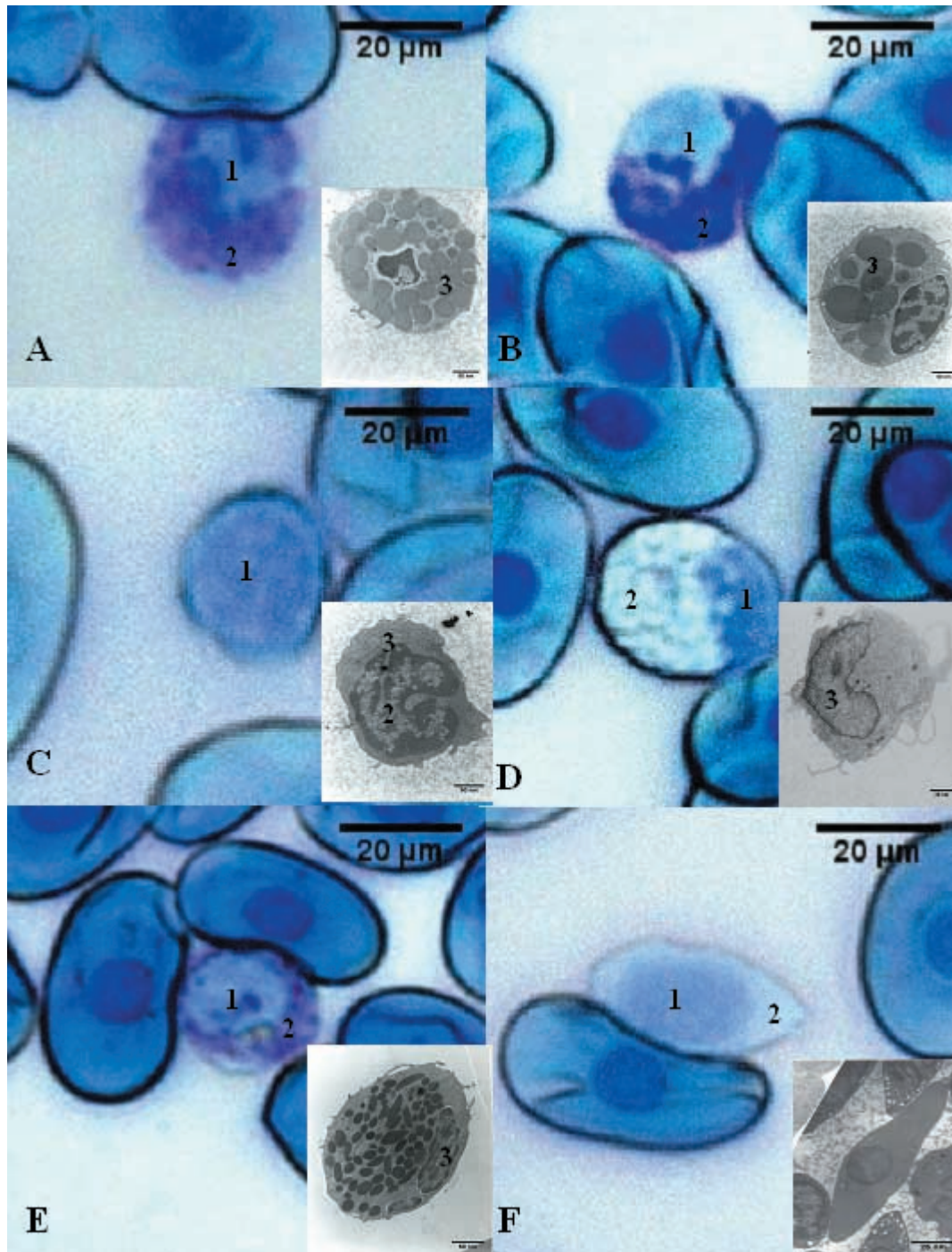


Fig. 1. A. Basophiles showing spherical shape with segmented nucleus (1), cytoplasm filled by many metachromatic granules (2) (400x of original magnification), granules with spherical shape under electron microscopy analysis (3) (2800x of original magnification). B. Eosinophils showing spherical shape and peripheral nucleus (1), cytoplasm filled by large granulus (2) (400x of original magnification). Granules with oval shape (3) (2500x of original magnification). C. Small lymphocytes with spherical shape and eccentric nucleus (1) filling almost all the cytoplasm (2) (400x of original magnification). However, when it is observed under electron microscope, the nucleus presented reniform aspect (3) (2500x of original magnification). D. Monocyte showing spherical shape with peripheral and reniform nucleus (1) and abundant cytoplasm (2) (400x of original magnification). Nucleolus under electron microscope (3) (2500x of original magnification). E. Heterophils (Neutrophils) showing spherical shape, central nucleus (1) and cytoplasm with elongated granules (2) observed under light microscope (400x of original magnification). However, when observed under electron microscope, it was found a peripheral and segmented nucleus (3) (2500x of original magnification). F. Thrombocytes showing an elliptic shape and nucleus with elliptic conformation too (1), and little quantity of cytoplasm (2) (400x of original magnification).

of basophiles, eosinophils, lymphocytes, monocytes, and heterophils (Oliveira *et al.*, 2000; Metin *et al.*, 2006). Studies involving ultrastructural citochemistry analysis performed in *Chrysemys dorbignih* specie of turtle, confirmed the existence of eosinophils and heterophils in blood samples and different morphological characteristics of these cellular types according to some variations (Azevedo & Lunardi).

The leukocyte identification is based on staining parameters and the morphology showed under light microscopy analysis, but it has some limits when this analysis is performed using leukocytes non granulocytic. Lymphocytes cells are in most cases classified as small, middle and large, and many authors relate the difficulty to analyze the differentiation of large lymphocytes in relation to monocytes or thrombocytes cells (Montali, 1988). Work, in 1998, analyzed the blood of *Chelonia mydas* specie, using sudan black B, periodic acid-schiff and toluidine blue, and cytochemistry reaction for acid phosphatase analysis and later identification of six types of leukocytes under electron microscope apparatus.

The results of the blood sample analysis stained by Leishmann, permitted to observe all leukocytes, being clear

that the differences between monocytes and thrombocytes cells. These results associated to the transmission electron microscopy analysis, permitted to observe the characteristic shape of the basophiles, eosinophils, and heterophils granules in more detail, and to affirm that the leukocyte morphology in *Phrynops hilarii* specie is very similar to the another species and according to morphological descriptions found in the literature (Ryerson; Oliveira *et al.*; Azevedo & Lunardi; Work *et al.*; Deen, 2006.). The Leishmann stain is used in most cases that the objective is to observe the hematological characteristics of the tissues in mammalian groups. Hughes *et al.*, 2003 evidenced very well each cellular type in this group, thus we can affirm that this type of staining is an excellent method for diagnosis in chelonian blood samples.

It was not observe any cellular type with similar characteristics to neutrophils in mammalian group. We believed, based on the data obtained in this study that the heterophils have a morphofuncional analogy with another neutrophils belonged to mammalian group. This conclusion is being supported in many recent studies found in the literature.

PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O. Caracterización morfológica de leucocitos circulantes en la sangre de la tortuga (*Phrynops hilarii*). *Int. J. Morphol.*, 25(4):677-682, 2007.

RESUMEN: La especie de tortuga *Phrynops hilarii* no ha sido aún bien descrita en la literatura. Fue propuesto en este estudio la caracterización de leucocitos de sangre de este animal coloreados con el método de Leishman y analizados con microscopías de luz y electrónica. No fue observado ningún tipo celular con características similares a los neutrófilos de mamíferos. Los resultados indican que los heterófilos tienen analogía morfofuncional con otros neutrófilos presentes en el grupo de los mamíferos. Esta conclusión es sustentada por varios estudios recientes encontrados en la literatura.

PALABRAS CLAVE: Tortuga; Morfología; Leucocitos; Sangre.

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