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# Micromorphometry, Cell Distribution and Ultrastructure of Some Immunopotent Leucocytes from Pronephric Kidney of *Channa punctatus* (Bloch)

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## Abstract

Leucocytes in the pronephric head kidney of five *Channa punctatus* (Bloch 1793), from wetlands near Kolkata, West Bengal, India, were studied through stained imprints on glass slides for light microscopy, and fixed thin sections for transmission electron microscopy. The morphology, shape and size of the leucocytes – neutrophils, eosinophils, large and small lymphocytes and macrophages – and their nuclei are described. The relative distribution of the cell types were 37-47, 4-7, 18-24, 17-30 and 9-16 %, respectively. The areas, in $\mu m^2$, of each cell type and its nucleus are given. Macrophages are monocyte-like with well developed Golgi complex, pigments and intracytoplasmic vesicles and vacuoles.

## Introduction

Parasitic infections, causing diseases in both wild and cultured fish, are a significant cause for concern. Immunopathology of fish demands correct identification of their immunopotent leucocytes. In the present work, we have studied, through light (LM) and transmission electron mi-

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croscopy (TEM), the leucocytes from the pronephric head kidney of *Channa punctatus* (Bloch 1793), an air breathing fresh water fish. The teleost head kidney is a major haemopoietic tissue (Ellis 1977; Bielek 1981).

Fishes encounter a variety of pathogens, parasites and pollution in the aquatic environment. The population of adult spotted murrel *C. punctatus*, fam. Channidae, previously abundant in the shallow wetlands of West Bengal, India is now dwindling aside from facing pollution and various disease problems in nature. Since it is important to establish a classification of leucocytes for histopathological examination of diseased fish and for experimental study of inflammation, detailed micromorphometry and relative abundance of the leucocytes of *Channa* species may give insight into the functions of these cells in the immune response of fish. It is now well accepted that in teleosts leucocyte subpopulation consists of granulocytes (neutrophils and eosinophils), lymphocytes, monocytes, macrophages and melanomacrophage cells. However, their identification, functions and relative contributions to specific and non-specific immune responses are still a matter of debate (Rowley et al. 1988; Hine 1992). Electron microscopy studies of leucocytes have been reported in various fish species. Most of these reports, however, deal with a limited type of cell and only a few aimed to classify leucocytes by electron microscopy (Weinreb 1963; Ferguson 1976; Morrow and Pulsford 1980; Barber et al. 1981; Roubal 1986). The purpose of the present study is to describe and attempt to understand the function of head kidney leucocytes in *C. punctatus.*

## Materials and Methods

Adult spotted murrels, *C. punctatus*, were collected from local point sources during summer of 2005 in and around the wetland areas near Kolkata, West Bengal, India. The fishes were transported live to The Department of Zoology, University of Calcutta, and were kept alive for one week in a glass aquarium (4′ x 1.5′ x 1.5′ ) with continuous aeration; water temperature was maintained at 24 ± 2 ºC. The fishes were fed *ad libitum* with *Tubifex* sp. and larvae of *Culex* sp. The specimen selected for study averaged 81.5 ± 2.24 g in weight and 19.7± 0.3 cm in length; all were more than one year old (1+ age group). Before collection of the haemopoietic tissue samples, each of the five fish sampled was wiped dry with a clean

towel, and anaesthetized by MS 222. Its round weight was measured to the nearest gram and its total length was measured to nearest centimeter. The ventral body wall was cut, the viscera exposed and head kidney was dissected out.

For light microscopy, air-dried head kidney tissue was imprinted on glass slides using 3-4 pieces from each fish. According to Ashley and Smith (1963), the tissue imprints were differentially stained in Benzidine (Graham-Knoll 1918) and counterstained in Giemsa (Mahajan and Dheer 1979a). The colour atlas of Dominguez and Villalobos (1947) was used to assess the colours of the stained cells. The stained imprints were examined using a Leica, DMR, DC 300 FX microscope and photomicrographs were taken. For morphometric analysis, the leucocyte populations from the pronephric kidney imprints of *C. punctatus* were measured using an ocular micrometer (LM magnification 10x100). The areas of the circular and oval leucocytes and their nuclei were calculated from $\pi r^2$ where r = half cell diameter, and $\pi ab$, where a and b = half of the axes a and b respectively.

For TEM analysis, pieces of head kidney tissue were washed in 0.1M phosphate buffer solution (pH 7.2) and fixed in 4 % gluteraldehyde solution (Karnovsky 1965), for 12 hours at 4ºC, followed by washing with freshly prepared buffer. The tissues were post-fixed in 1% aqueous osmium tetroxide ($OsO_4$) solution for 2 hours at 4ºC. After repeated washing in 0.1M phosphate buffer, tissues were dehydrated in ascending grades of ethanol up to 100%, infiltrated and embedded in Araldite CY 212 (TAAB, UK). Ultrathin sections (60-80 nm) were cut on an ultramicrotome (ultracut E, Reichert Jung). The sections were mounted on meshed copper grids and stained in aqueous uranyl acetate (10 min) and lead citrate (10 min). The sections were examined on a Morgagni 268D Transmission Electron Microscope (Fei Electron Optics, The Netherlands) at accelerating voltage of 80 KV, in All India Institute of Medical Sciences, New Delhi.

## Results

### *Light microscopy*

The following leucocytes were studied from the pronephric kidney imprints. The characteristic features of these leucocytes including mean cell area (C), cell shape, percentage distribution, mean nuclear area (N), shape of the nucleus and C-N ratio are shown in table 1.

Table 1. Characteristics of the immunopotent leucocytes from head kidney imprints of *C. punctatus*

| Cell type | Shape | | Area ($\mu m^2$) (mean area ± S.E.) $n_1$ =25 | | Ratio C/N | Percentage Distribution $n_2$= 5 |
|---|---|---|---|---|---|---|
| | Cell | Nucleus | Cell (C) | Nucleus (N) | | |
| Neutrophil | Round | Round/ Kidney-shaped | 103.82 ± 2.50 | 33.40 ± 1.78 | 3.11 | 37-47 |
| Eosinophil | Round/ Oval | Spheri-cal/Irregular | 89.66 ±3.40 | 24.33 ±0.82 | 3.67 | 4-7 |
| Large Lympho-cyte | Round | Round | 61.95 ±1.83 | 31.86 ±1.30 | 1.94 | 18-24 |
| Small Lympho-cyte | Round | Round | 24.10 ±0.085 | 13.84 ±0.54 | 1.74 | 17-30 |
| Macro-phage | Circu-lar/ Irregular | Flattened/ Strongly eccentric/ Horseshoe-shaped | 163.17 ±3.18 | 47.46 ±1.22 | 3.44 | 9-16 |

$n_1$= Number of cells observed, $n_2$= Number of fish observed

*Neutrophils*

Among the granulocytes, neutrophils (Plate 1, Figure 1) were present in large numbers representing about 37-47 % of the total leucocytes in the pronephric kidney. The average cell size was 103.82 ± 2.50 $\mu m^2$ and its nuclear area was 33.40 ± 1.78 $\mu m^2$. These cells are mostly round in shape and contain either a centrally placed or eccentrically placed mono-, bi-, or multi-lobed nucleus of various shapes. The heterochromatin in these nuclei tended to be condensed; no nucleolus was evident in the mature cell. The cytoplasm contained numerous bluish-stained granules and vacuoles were infrequent.

*Eosinophils*

The eosinophils (Plate 1, Figure 1) represented about 4 -7% of the total leucocytes. The cells were round to oval, with an average size of 89.66 ± 3.40 $\mu m^2$. The average nucleus, 24.43 ± 0.82 $\mu m^2$, was eccentrically placed, generally single-lobed, spherical but bi-lobed nuclei with

irregular shape were also found. The nucleus stained a deep purple with densely packed chromatin. A small number of pinkish indistinct granules were present in an otherwise neutral-stained cytoplasm. Vacuoles were frequent in the cytoplasm.

*Lymphocytes*

Two classes of lymphocytes (small and large) were seen in *C. punctatus*. Small circular lymphocytes represented about 17-30% of the leucocytes. The large lymphocytes constituted 18-24%. Both lymphocytes had an almost spherical, dark purple nucleus, outlined with small traces of a faint pinkish rim of basophilic cytoplasm and few or no pseudopodia. In *Channa* sp., the small lymphocytes predominated (Plate 1, Figure 2). Pseudopodia were not frequent and nuclear clefting was occasionally observed. The average cell area and nucleus area of small lymphocytes were 24.10 ± 0.85 $\mu m^2$ and 13.84 ± 0.54 $\mu m^2$ respectively and those of large lymphocytes were 61.95 ± 1.83 $\mu m^2$ and 31.86 ± 1.30 $\mu m^2$ respectively.

*Macrophages*

The macrophages (Plate 1, Figure2) also showed a range in size and morphology. The macrophages represented 9-16% of the total leucocytes. Identifiable macrophages were the largest cells in the kidney imprints, average cell size 163.17 ± 3.18 $\mu m^2$ and nuclear size of 47.46 ± 1.22 $\mu m^2$. The nucleus ranged from flattened and strongly eccentric to indented, horseshoe-shaped, as in classical macrophages. The cytoplasm was very faintly stained and appeared non-granular. The nucleus stained deep purple. These cells were easily recognized by vacuoles in the basophilic cytoplasm. However, intermediate morphologies were found and it was often not clear whether such cells were monocytes or macrophages.

## *Electron microscopy*

The TEM micrographs of the various leucocytes from the head kidney of *C. punctatus* are shown in Plates 2 and 3.

*Neutrophils*

The cell outline was often irregular but pseudopodia were absent. A few pinocytic vesicles were found at the periphery of the cytoplasm. The nucleus was occasionally bilobed (Plate 2, Figure 2) and kidney shaped. Trilobed nuclei were rare. The nucleus, irregular in outline, was not observed to be multilobed. The nuclear chromatin was dense and patchy in distribution. Nucleoli were very rare. The cytoplasm contained several mitochondria, plenty of smooth endoplasmic reticulum (SER), rough en-

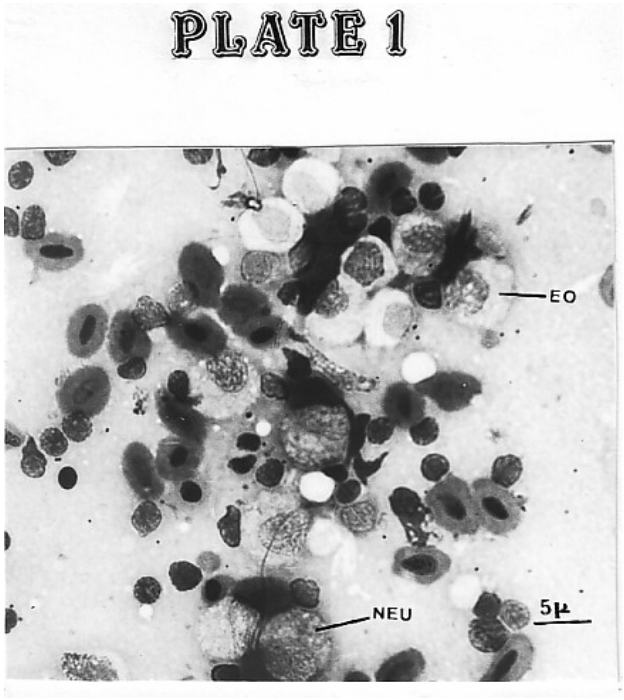



Fig. 1. An imprint from head kidney of .*C. punctatus*, showing neutrophil (NEU) and eosinophil (EO) [x 1,600] leucocytes

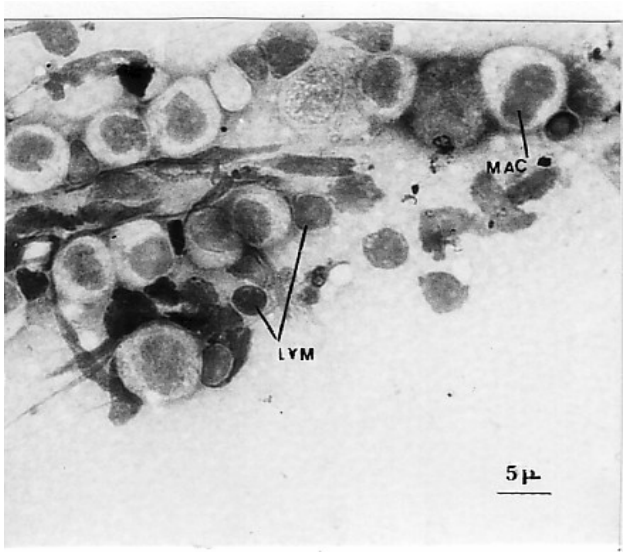
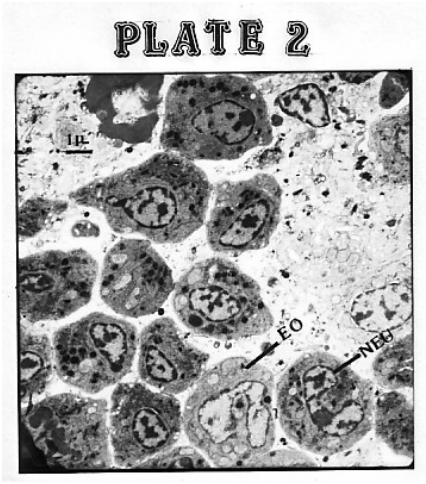



Fig. 2. An imprint from head kidney of *C. punctatus*, showing lymphocyte (LYM) and macrophage (MAC) [x 1,600] leucocytes




Transmission electron micrographs of leucocytes from head kidney of *C. punctatus*

Fig. 1. Neutrophil (NEU) and eosinophil (EO) [x 4,500]

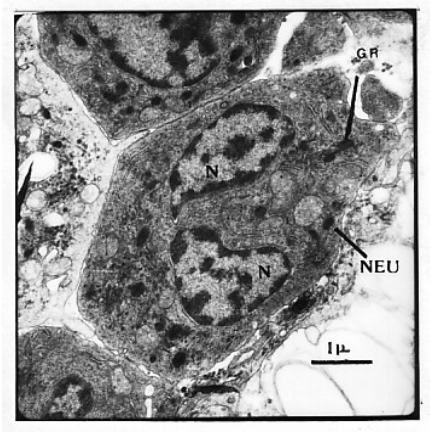



Fig. 2. Neutrophil (NEU) with granules (GR) present in the cytoplasm and characteristic bi-lobed nucleus (N) [x 11,400]

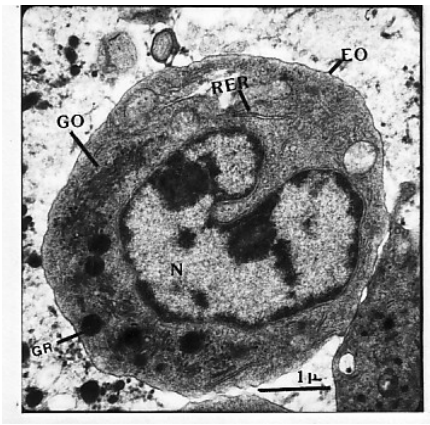



Fig. 3. Eosinophil (EO) showing characteristic large nucleus (N), Golgi apparatus (GO), Rough Endoplasmic Reticulum (RER) and granules (GR) [x 14, 400]

doplasmic reticulum (RER) located near the nucleus or at the periphery of the cytoplasm. The Golgi complex was not prominent. A small number of free ribosomes were scattered throughout the cytoplasm and clusters of glycogen granules were occasionally observed. The cytoplasm was distinguished by its numerous large, round to elongated, light and dark granules (Plate 2, Figure 2). The content of these granules showed varying degrees of organization, all surrounded by a limiting membrane. The cytoplasm of the neutrophil showed much granularity due to the presence of ribosomal materials.

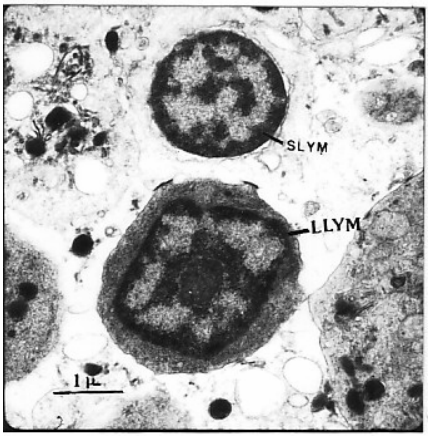
*Eosinophils*

Eosinophils were round cells with an eccentric nucleus, which was always round to oval and rarely lobed (Plate 2, Figure 3). The nuclear membrane was prominent, and nucleolus was very rare. The cell outline was often irregular. In the cytoplasm there were Golgi complex, mitochondria and RER. A moderate number of free ribosomes were scattered throughout the cytoplasm. A considerable amount of fine cytoplasmic vesicles were also observed at the periphery of the cytoplasm. A few large characteristically round or oval granules were seen in the cytoplasm. These granules, enclosed by a limiting membrane, were uniformly distributed in the cytoplasm. The granules, having a dark content with often light zones (Plate 2, Figure 3), were not separated by membrane.

*Lymphocytes*

This was the smallest of the four leucocytes and was characterized by its large central nucleus surrounded by a thin rim of cytoplasm (Plate 3, Figures 1 and 2). The plasma membrane was plicate and with occasional small pseudopodia, in some cases with microvilli. The nucleus was often indented or clefted (Plate 3, Figure 2). Nuclear chromatin was dense, but was mainly peripheral in distribution. The nucleus was dark with a dense heterochromatin area. Ultrastructurally, in small lymphocytes (Plate 3, Figure 1), the nucleus contained relatively few heterochromatin. Small vesicles, limited amounts of RER, and many scattered ribosomes and few round or oval, sometimes ellipsoidal mitochondria comprised the bulk of cytoplasm. The SER was rare and glycogen granules were few. In the narrow cytoplasm there were a few round, dense bodies that were enclosed by a limiting membrane and resembled neutrophil granules. The Golgi complex was poorly developed.

PLATE 3




Transmission electron micrographs of leucocytes from head kidney of *C. punctatus.*

Fig. 1. Small lymphocyte (SLYM) and large lymphocyte (LLYM) with characteristic large round nucleus covering most of the cell area [x 14,400]

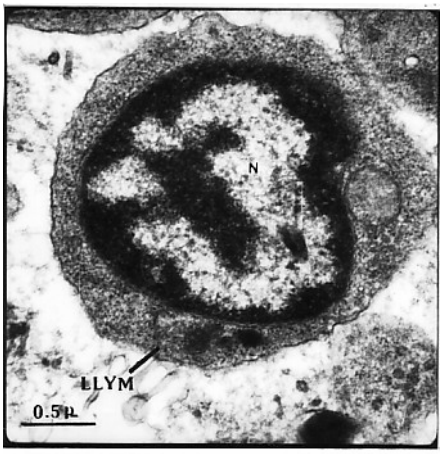



Fig. 2. Large lymphocyte (LLYM) with characteristic large nucleus (N) and cytoplasma rim [x 29,100]

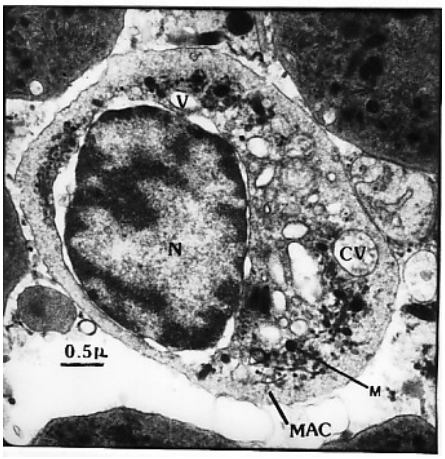



Fig. 3. Macrophage (MAC) showing eccentric nucleus (N), many cytoplasmic vesicles (CV), vacuoles (V) and melanin granules (M) [x 16,320]

*Macrophages*

The macrophage of *Channa* sp. was slightly larger than the neutrophil, due mainly to its irregular outline. Its shape was round to oval (Plate 3, Figure 3). The large central to eccentric nucleus was without lobes and with a rim of heterochromatin and clumped heterochromatin throughout the nucleus. There may be a large nucleolus. The nucleus was electron lucent because of a wide euchromatin area. The cytoplasm was replete with lysosomes, oval mitochondria, RER, well-developed Golgi complex, pigments and other cellular debris. Moderate numbers of free ribosomes were scattered throughout the cytoplasm. Most of the macrophages had many vesicles containing melanin (Plate 3, Figure 3), lipofuscin and haemosiderin. These pigments were observed within a lysosomal apparatus or lying freely within the cytoplasm. The vesicles were round, oval or tubular and have an electron-opaque/dense content. Many intracytoplasmic vacuoles were also observed at the periphery of the cytoplasm (Plate 3, Figure 3).

## Discussion

A comprehensive understanding of the identity of leucocytes of individual fish species is necessary prior to any significant advance in clarifying their immunological role. With the help of morphological, morphometric, cytochemical and autoradiographic techniques, Mahajan and Dheer (1979b) characterized and identified different cell types in the peripheral blood of *C. punctatus*. In the present study, leucocyte cell populations from the haemopoietic tissues of *C. punctatus* were observed and analyzed through LM and TEM. Over the last two decades, a number of ultrastructural descriptions have been made on the peripheral blood leucocytes of various fish species (Ellis 1976; Blaxhall 1983; Cenini 1984; Parish et al. 1986) as well as on the major haemopoietic organs of fishes (Bodammer et al. 1990; Quentel and Obach 1992; Dash et al. 2003). However, there exist conflicting reports that may be attributed to the inherent variability in individual fish species. In *C. punctatus*, neutrophils and eosinophils could be identified among the granulocytes based on their granular morphology. Watanabe et al. (1967) reported that parallel linear structures were present in the human neutrophils. Similar bodies described as fibrillar structures in fish neutrophils were also reported (Suzuki 1986; Hine and Wain 1988). Fujimaki and Isoda (1990) described type I granules

in gold fish neutrophils similar to such stratified parallel linear structures. The structural similarity has been observed in the granules of neutrophils of *Channa* sp. Fish eosinophils, on the contrary, possess homogenous granules without any specific internal structures in various fish species. However, there are a few studies describing stratified, crystalloid granules in fish eosinophils (Fujimaki and Isoda 1990). In our study, no specific internal structure could be identified. Lymphocytes of the lymphoid series were also identifiable in *C. punctatus*. Small and large lymphocytes could also be separated by TEM in our study (Plate 3, Figure 1). The lymphocytes could be differentiated by a lack of dense bands of heterochromatin as seen in the thrombocyte nucleus, but instead having a nucleus with a less dense rim and scattered patches of heterochromatin. In the present study, we did not perform any histochemical and immunological test to confirm the distinction of the agranulocytes. Earlier studies on phagocytic processes *in vitro* indicated that of all the cells macrophages are the most efficient phagocytic cells (Verburg van Kemenade et al. 1994). Macrophages from pronephric cells have been identified as monocyte-like cells based on their size and relatively small number of lysosomes. Appropriate identification of fish leucocytes is a foremost requisite for immunopathological investigations. It is hoped that the descriptions presented in the present study clarify some of the confusion.

## Conclusion

Until recently, a comparative pathology of fish has not been considered as a justifiable study in the assessment of fish health, probably due to paucity of information on the subject. In our study structural details of the various leucocytes of *C. punctatus* have been presented. It is hoped that the present study will contribute to a better understanding of the ultrastructural pathology of this fish species, whose population decline may be caused by pollution, microorganisms or parasites in their shallow natural habitat.

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