ULTRASTRUCTURE OF LEUKOCYTES FROM THE PERIPHERAL BLOOD OF DUTTAPHRYNUS HIMALAYANUS (GÜNTERH)

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ABSTRACT

Duttaphrynus himalayanus is an atypical amphibian species, adapted to high altitude and cold climatic conditions of Eastern Himalaya. The peripheral blood leucocytes from D. himalayanus were studied by transmission electron microscopy. The ultra structure and sub cellular organizational architecture of the leukocytes appears to be very similar to mammalian leukocyte and accordingly have been characterized into five different types.

Key words : Ultrastructure, Duttaphrynus himalayanus, Transmission electron microscopy, WBC, Lymphocytes.

Amphibians have an evolutionary history of more than three hundred million years (Anderson et. al., 2008). Duttaphrynus himalayanus is an inimitable amphibian species, adapted to cold climatic conditions of the Eastern Himalayas and appears to differ from the other members of Bufonidae found in the plains of West Bengal, India. They have a very restricted distribution and are constrained to the hills of Darjeeling and Sikkim in India and in parts of southern China. This species appears to have evolved in relative isolation in restricted pockets of the Himalayas, 5000ft above sea level, probably since the formation of the Himalayas and therefore becomes an ideal species for investigation of the adaptation of the immune system to varied climatic conditions.

Immune system of amphibians, especially the African clawed frog, Xenopus laevis have been described earlier (Du Pasquier et al., 1989; Forbes, et al., 2006; Gentz, 2007), but absolutely nothing is known about the immune system of the D. himalayanus that presents a special feature of adaptation in cold climatic conditions in spite of being a cold blooded animal. In an attempt to study the evolution of immune system in anurans, wild Duttaphrynus himalayanus has been selected as the model for the present investigation especially because this animal has evolved in relative isolation since the formation of the Himalaya. In higher vertebrates, Neutrophils, Eosinophils, Basophils and Monocytes are generally associated with the innate immune response, providing protection against a wide range of foreign antigens and parasites whereas, lymphocytes (Pettey et al. 1981; Muller et al. 1975; Blomberg et al. 1980; Hirsch and Fedorko, 1968) and macrophages (Klaur et al.,1980; Wolfgang et al., 1982) constitute the arms of the adaptive immune system and play an extremely important role in the process of eliciting an effective adaptive immune response. Scanning electron microscopic observation of the leukocytes from D. himalayanus allowed to categorize them into five different types of leukocytes (Das and Sarkar, 2005; Bhattacharjee and Das, 2008) as found in mammals. In the present

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investigation, the ultrastructure and the subcellular organelles of the peripheral blood leukocytes are described as seen by transmission electron microscopy.

**MATERIALS AND METHODS**

*Animal Model – Duttaphrynus himalayanus* that falls under the “least concerned” category of the IUCN red list, 2008* 17 is the model of the present investigation. Adult, healthy individuals were captured from the wild and were maintained in the laboratory under proper hygienic conditions and fed with house flies and chopped chicken and goat liver. The animals were sacrificed following the guidelines and approval of the Animal Ethical Committee of the institute. Approximately 0.5-1.0 ml blood was collected by ventricular puncture in tubes containing EDTA (Sigma, USA).

*Isolation of Leucocytes –* The leucocytes were freed from red blood cells by centrifugation over isopycnic percoll density gradient as described earlier (Das and Sarkar, 2005). The cell fractions were collected from the 65% percoll interphase and then washed twice with cold amphibian phosphate buffer saline (APBS, pH – 7.2) to remove the percoll. The viability of the cells was tested by trypan blue exclusion method and finally suspended in L-15 medium at a concentration 2x10^6 cells/ml.

*Medium –* Leukocytes were suspended in Leibovitz-15 (L-15) medium (Hi-Media, Bombay), supplemented with glutamine, 1.25 x 10^-5 M HEPES buffer, 200 mg NaHCO₃/100 μl, 100 U of penicillin/ml, (Sigma, USA); 100 μg/ml streptomycin, (Sigma, USA); 50 μg/ml Nystatin, (Sigma, USA); and 10% heat inactivated sterile fetal calf serum.

*Transmission Electron Microscopy (TEM) –* For TEM study, leucocytes were fixed with 2.5 % gluteraldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 30 minutes at room temperature (RT). Excess fixative was removed by repeated centrifugation at 200 g with 0.1 M phosphate buffer (PB). Post fixation was made in 1% Osmium tetroxide for 15 minutes at RT and then suspended in 0.25% uranyl acetate in 0.1 M PB (pH 6.3) for 15 minutes followed by washing with distilled water by centrifugation. Cell pellet was then dehydrated with acetone and infiltrated with a 1:1 mixture of dry acetone and embedding medium for 2 hours. Finally the samples were embedded in embedding medium containing 10 ml araldite CY212, 10 ml Dodecenyl Succinic Anhydride (DDSA), 0.4 ml 2, 4, 6 tridimethylamino methyl phenol (DMP-30) and 1 ml plasticizer (Dibutyl phthalate) using beem capsules. Polymerization of the resin was allowed for 24 hours at 60°C. Ultra thin silver sections of 60-90 nm were obtained from Ultracut UCT, Reichert Division, Leica Ultramicrotome. The sections were taken on specially made copper grids and stained with a saturated solution of uranyl acetate. After washing briefly with 0.02 M sodium hydroxide followed by 2 lots of double distilled water the grids were dried. Then the grids containing sections were examined and photographed in a CM-10, Philips Transmission Electron Microscope at 60, 80 and 100 KV accelerating voltages at the Sophisticated Analytical Instrumentation Facility (DST), Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India.

**RESULTS AND DISCUSSION**

Isolation of the amphibian leukocytes from the peripheral blood was a major obstacle, which was overcome by adopting a new method of cell fractionation over 65% percoll gradients (Das and Sarkar, 2005). This single step method of isolation yields almost 99% leukocytes and nontoxic (Pertoft et al., (1977) Nathanel et al., (1981) Opstelten et al., 1982) nature of percoll allowed 98% recovery of viable cells (Das S K and Chakravarty, 1998). Leukocytes so isolated were used for TEM study.

TEM observations of the *D. himalayanus* leukocytes were corroborated with the electron microscopic slides of mammalian leukocytes which revealed close similarity except that amphibian leukocytes are slightly larger than their mammalian
The leukocyte population could easily be differentiated into granulated and agranulated cells. The granulated cells possess several different types of secretory granules distributed throughout the cytoplasm. Apart from the normal cell organelles like the mitochondria, Golgi complex and endoplasmic reticulum; lysosomes and phagolysosomes are sometimes seen. The granulated cells could further be distinguished into three types on the basis of their size, cell membrane architecture, and cytoplasmic organelles and frequency of observations.

Granulated cells Type IA – The cells grouped under this category were most frequently observed under the microscope. Their size varied from 8 to 12 μm in diameter and was comparatively larger than other cell types. The cell membranes were relatively smooth as seen in Fig. 1A, but blunt pseudopodia were sometimes noticed. Long cytoplasmic extensions were never present. The nucleus was polymorphic with heterochromatin concentrated at the periphery of the nucleus and euchromatin at the center. Significant number of electron dense granules was found distributed throughout the cytoplasm. The larger electron dense granules seem to be primary lysosomes (PLy) which are referred as azurophile granules (Az) in light microscopy (Cieutat et al., 1998). The size of the granules and vacuolation in the cytoplasm were similar to the neutrophils of mammals. Few phagocytic vesicles (PV) at different stages of maturation were also visible. Endoplasmic reticulum (ER), mitochondria (M) are as usual. From the frequency of observations, nature of granules and nuclear architecture, the cells seem to be Neutrophils.

Granulated cells Type IB – The cells placed under this group of were characterized on the basis of presence of distinct electron dense granules in the cytoplasm (Fig. 1B). The granules contain crystalline proteins that are clearly visible. The granules appear to contain major basic proteins as seen mammalian eosinophils (Gleich et al., 1973) that form crystals. The larger granules are primary lysosome. Phagocytic vesicles are sometime observed. The nucleus is polymorphic with electron dense region at the periphery. The cell membranes are highly irregular with blunt pseudopodia (BP), an indication of the phagocytic nature of the cells. The size of these cells is relatively smaller than the neutrophils. The characteristic nature of the granules and cell morphology indicates them to be eosinophils.

Granulated cells Type IC – The presence of this type of cells was very rare in the sections observed. These granulocytes had a diameter similar to the eosinophils (Fig 1C). The cell membrane is relatively smooth but blunt pseudopodia were present. The cytoplasm contains large electron dense granules, likely to be primary lysosomes of various shapes and sizes, a feature not observed in other cell types. However, the granules did not have any crystalline structures. Vacuoles containing ingredients within were present, which are likely to be phagocytic vesicles. Small glycogen granules (Gl) are also present in the cytoplasm. In all probability

<p>| Table 1: Total Count and Differential count of blood leukocytes of D. himalayanus. |
|--------------------------------------|--------------------------------------|--------------------------------------|</p>
<table>
<thead>
<tr>
<th>Cell types</th>
<th>Number of cells per cubic mm of blood</th>
<th>Male</th>
<th>Female</th>
<th>Relative abundance% (Mean ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>5000-8000</td>
<td>4000-8000</td>
<td>(48.7± 3.5)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>200-600</td>
<td>200-600</td>
<td>(2.7±0.9)</td>
<td></td>
</tr>
<tr>
<td>Basophils</td>
<td>100-550</td>
<td>100-550</td>
<td>(2.1±0.6)</td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>1500-2500</td>
<td>1400-2500</td>
<td>(16.4±1.6)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2500-3500</td>
<td>2500-3500</td>
<td>(28.2±2.8)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>13000-14000</td>
<td>12000-14000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*Values are of 10 replications).
these cells were basophils. Eosinophils and basophils which could not be distinguished by scanning electron microscopy (Das and Sarkar, 2005) were clearly distinguishable in the TEM pictures from the disposition of crystalline proteins within the granules of eosinophils.

Agranulated Cells or Type II cells – The cytoplasm of these cells were free of granules. Only a few secretary vesicles were seen. On the basis of the cell membrane morphology and nuclear organization, the cells were further differentiated into two types.

Agranulated Cells: Type II A & II B – This group of cells was relatively large with irregular cell membrane. Cytoplasmic granules like primary lysosomes (Ply), Mitochondria (M), Golgi complex (Go), Glycogen Granules (Gl), etc were present in the cytoplasm. The nucleus was polymorphic, typically horse shoe shaped (Fig 2A) with irregular nuclear membrane just like human monocytes (Hirsch and Fedorko, 1968). Some of the cells exhibited extensive pseudopodia like projections (P) which are probably macrophages (Fig 2B). Along with secondary lysosomal vesicles, cytoplasmic phagocytic bodies (PB) were also visible, clearly indicating active phagocytosis activity (Fig 2B). Thus, these cells seem to be active macrophages.

Agranulated Cells: Type II C – The group of cells categorized under this group had cell diameter ranging from 7 to 9 microns (Fig 2C). The cells have a spherical appearance with smooth cell membrane.

Fig. 1: Ultrastructural details of the granular leukocytes D. Himalayanus. (a) Type IA: Neutrophil; (b) Type IB: Eosinophil; (c) Type IC (Basophil).
No pseudopodia or cytoplasmic extensions were visible. The nucleus was always rounded smooth and never lobed like the nucleus of other white blood cells. The nucleus occupies most of the cell volume and sometime is slightly eccentric in position. The euchromatin primarily occupied the central region and the heterochromatin at the periphery. The cytoplasmic material exists as thin rim lining the nuclear membrane and included few mitochondria but no lysosome or other types of granules were seen. Undoubtedly, these cells are lymphocytes.

Total white blood count of *D. himalayanus* is shown in Table 1. The neutrophils were most frequently observed followed by the lymphocytes and then monocytes. The number of eosinophils and basophils were less frequently seen in all the slides. The frequency at which the different cell types observed under transmission electron microscopy corroborated with the light microscopic findings. Interestingly the relative ratios of each cell type were similar to mammals but the total count of blood leukocytes was higher than that of human. It may also be noted that the sizes of the amphibian blood cells are also slightly larger than their mammalian counterparts.

**Fig. 2**: Ultrastructural details of the agranular leukocytes *D. Himalayanus*. (a) Type IIA: Monocyte; (b) Type IIB: Macrophages (c) Type IIC: Lymphocyte [azurophile granules (Az); mitochondria (M); glycogen granules (Gl); phagocytic vacuoles (PV); lysosomes (Ly); primary lysosomes (PLy); golgi complex (Go); pseudopodia (P); blunt pseudopodia (BP); cytoplasmic crystalline granules (Gr)].
The vivid similarity between the present amphibian and mammalian leukocytes indicates that the amphibians during their transition from water to land millions of years ago designed and developed immune strategies that still carry relevance and most likely the same lineage of the primitive leukocyte architecture exists in all vertebrates. For example, macrophages from different organisms including mammals have been shown to possess scavenger receptors that facilitate the recognition non-self ligands. However, biochemical analysis of the cytoplasmic granules is required to assess whether the granules carry the same lethal components to destroy foreign elements as in mammals. There is little doubt that the innate and adaptive immunity has undergone drastic transformation in mammals, but the basic plan still remains the same.

The transmission electron microscopic observation of the leukocytes will immensely help to carry forward the process of characterization of the immuocompetent cells of *D. himalayanus*, and to understand how the immune system of this unique species is adopted to combat various pathogenic agents at high altitude and low temperatures.

**ACKNOWLEDGEMENT**

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**REFERENCES**


Global amphibian assessment report 2008, [Species Survival Commission (SSC), International union for Conservation of Nature and Natural Resources (IUCN)].


