Journal of Animal and Veterinary Advances 7 (7): 847-853, 2008
ISSN: 1680-5593


# Hematology, Cytochemistry and Ultrastructure of Blood Cells in Clouded Leopard (*Neofelis nebulosa*)

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**Abstract:** Basic hematology, cytochemistry and ultrastructural features of blood cells in the clouded leopard (*Neofelis nebulosa*) are described for the first time. There was no significant difference ($p<0.05$) of all hematological values between genders. Unique morphologies and cytochemical profiles of blood cells were identified. The morphologies of leukocytes were similar to those of domestic and some wild cats; the fishing cat and the flat-headed cat, except for eosinophil and basophil. The large round shaped granules of eosinophils were prominent and were not stained with any cytochemical stains, while basophils were easily observed and displayed fine dull blue-grey granules. This study provides a guideline for identification of blood cells in the clouded leopard which will be useful for health management in this endanger species and contributes to hematological resources in wildlife animals.

**Key words:** Blood cells, clouded leopard, cytochemistry, hematology, ultrastructure

## INTRODUCTION

The clouded leopard is the one of nine native wild cats found in Thailand (Lekagul and McNeely, 1988). It stands alone in its own genus as *Neofelis nebulosa*, literally the new cat with a cloudy pelt. Because of the beautiful pattern of its fur, many of the cats have been hunted for the illegal wild animal trade. Today, the clouded leopard is listed as an endangered animal (McNeely, 2000).

To conserve the endanger species, the clouded leopard breeding program was established at Khao Kheow Open Zoo, Thailand, in 2002. Hematology serves as a screening procedure to assess general health and the ability to fight infection and also provides information for patient education or diagnosis (Mills, 1998). Cytochemical staining is useful for the characterization of undifferentiated blood cells in humans and animals (Jain, 1986). Ultrastructural examination of leukocytes provides benefit in the identification of higher resolution organelles and granules (Steffens, 2000). However, basic hematological values, cytochemistry and ultrastructure of blood cells in this species have not been described.

The current study provides the first details of hematologic data, cytochemical properties and morphologies of blood cells in the clouded leopard. It describes individual cells via light microscopy, scanning (SEM) and transmission electron microscopy (TEM) and compares to the blood cells of domestic and those of some wild cats.

## MATERIALS AND METHODS

**Collection and analysis of blood samples:** Blood samples of seventeen clinically healthy clouded leopards in Khao Kheow Open Zoo, Thailand were collected from the femeral vein. Two milliliters of each sample were collected in EDTA.

The completed blood count (CBC) was performed using an automated cell counter (Baker 9110; BioChem ImmunoSystem, U.S.). Two direct blood smears from each sample were stained with Wright-Giemsa (WG) and Wright (W) stains (Jain, 1986). The diameters of each

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blood cells type were randomly measured and at least 200 leukocytes were differentiated. For a reticulocyte count, a wet preparation of new methylene blue (NMB) stained smear was applied. The percentage of reticulocyte was determined in 1000 red blood cells.

Cytochemical characteristics of blood cells were evaluated using an air-dried fresh blood smear. Blood cells were stained with Peroxidase (PER), Sudan black B, α-naphthyl acetate esterase (ANAE), Periodic acid-Schiff (PAS) (Jain, 1986) and β-glucuronidase (β-glu) (Hayhoe and Quaglino, 1980). Positive-and negative-stained cells were differentiated by counting 500 cells on each stained smears.

For ultrastructural features of each cell type, blood samples from 4 clouded leopards were processed as described previously (Salakij *et al*., 2002). The brief details were as follows.

With TEM, buffy coats from blood samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, at 4°C for 24 h, postfixed in 1% osmium tetroxide, dehydrated by acetone series and embedded in Spurr's epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Jeol JEM-1230 transmission electron microscope. Identification of each cell type was based on the relative number, size, shape, distribution of granules and nuclear appearance.

With SEM, 2 drops of fresh blood were fixed in 1.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, at 4°C overnight. Specimens were dehydrated in a graded series of acetone, then coated with gold by sputtering unit and viewed with a Jeol JSM-6460 LV scanning electron microscope. Each cellular detail was evaluated by fine surface features as described elsewhere (Nopanitaya and Somana, 1988).

**Statistical analysis:** Means and standard deviations of each hematological parameter and blood cell diameter were tested by analysis of variances (SPSS for Window version 11.5). Significant differences ($p<0.05$) between means of each parameter between sexes were determined by independent sample Mann-Whitney *U*-test.

## RESULTS

There was no significant difference of all hematological values between genders (Table 1). Blood cell diameters were measured and calculated (Table 2). Cytochemical profiles of each blood cell were summarized and compared with those previously published for fishing cats and flat-headed cats (Table 3). The morphology, cytochemistry and ultrastructures of individual blood cells were evaluated, as described in the study.

Table 1: Comparative hematology between the male and the female clouded leopards

| Parameters | Male (n=8) | Female (n=9) | Total (n=17) |
|---|---|---|---|
| PCV (%) | 35.2±4.9* | 37.2±4.5 | 36.3±4.700 |
| Hb (g $dL^{-1}$) | 11.3±1.200 | 11.7±1.2 | 11.5±1.200 |
| RBC ($\times 10^6$ μL) | 6.0±1.000 | 6.5±0.9 | 6.2±0.900 |
| MCV (fL) | 59.6±8.400 | 57.9±6.2 | 58.7±7.100 |
| MCH (pg) | 19.2±3.300 | 18.1±1.0 | 18.7±2.400 |
| MCHC g $dL^{-1}$) | 32.4±4.000 | 31.7±3.8 | 32.0±3.800 |
| WBC ($\times 10^3$ $\mu L^{-1}$) | 14.85±3.11 | 12.52±4.34 | 14.30±4.93 |
| Bands ($\times 10^3$ $\mu L^{-1}$) | 0.13±0.13 | 0.22±0.22 | 0.24±0.24 |
| Segmented neutrophil ($\times 10^3$ $\mu L^{-1}$) | 10.80±2.24 | 8.39±2.78 | 10.01±3.69 |
| Eosinophils ($\times 10^3$ $\mu L^{-1}$) | 0.87±0.39 | 0.93±0.71 | 0.90±0.56 |
| Basophils ($\times 10^3$ $\mu L^{-1}$) | 0.12±0.12 | 0.54±0.54 | 0.36±0.36 |
| Lymphocytes ($\times 10^3$ $\mu L^{-1}$) | 2.47±1.23 | 2.21±1.76 | 2.38±1.48 |
| Monocytes ($\times 10^3$ $\mu L^{-1}$) | 0.46±0.46 | 0.27±0.27 | 0.40±0.40 |
| Bands (%) | 0.9±0.900 | 1.7±1.500 | 1.5±1.500 |
| Segmented neutrophil (%) | 72.9±6.700 | 68.3±10.00 | 70.5±8.500 |
| Eosinophils (%) | 6.4±3.600 | 6.7±4.500 | 6.3±4.000 |
| Basophils (%) | 0.9±0.900 | 3.9±3.900 | 2.5±2.500 |
| Lymphocytes (%) | 16.0±6.100 | 16.9±9.500 | 16.3±7.800 |
| Monocytes (%) | 2.9±2.900 | 2.1±2.100 | 2.6±2.600 |
| Reticulocytes (%) | 0.1±0.100 | 0.1±0.100 | 0.04±0.04 |

*mean±SD

Table 2: Blood cell diameters in micrometer of the clouded leopards

| Cell type | Number | Diameters (μm) |
|---|---|---|
| Red blood cells | 1400 | 6.1±0.2* |
| Bands | 30 | 11.4±1.5 |
| Segmented neutrophils | 500 | 11.7±0.8 |
| Eosinophils | 50 | 12.6±1.2 |
| Basophils | 34 | 13.6±1.3 |
| Lymphocytes | | |
| Small | 105 | 8.6±0.3 |
| Medium | 177 | 10.8±0.3 |
| Large | 43 | 14.4±0.7 |
| Monocytes | 30 | 13.4±1.0 |

*mean±SD

**Erytrocytes:** Erythrocytes were large 6.1 μm mean diameter and were slightly variable in size. The rouleaux formation was frequently observed. Reticulocyte percentages were narrow ranges (Table 1). They were negative for all cytochemical stains.

**Neutrophils:** Neutrophils were the most prevalent leukocytes (Table 1). The average size of a segmented neutrophil was 11.7 μm, while the band form was 11.4 μm in diameter (Table 2). With WG and W, the constricted nuclei with faintly pink-bluish stained cytoplasmic granules were seen (Fig. 1a-b). Three to six percentages of neutrophils from the females revealed sex chromatin lobe. They were strongly positive for PER, SBB and PAS as the yellow, black and magenta granules (Fig. 1c, d and g), but weakly positive for ANAE and β-glu (Fig. 1e-f). With a scanning electron microscope (SEM), they were round and endowed with numerous microvilli (Fig. 2a).

Table 3: Cytochemical staining reactions of various blood cells in clouded leopards as compared to those previously published for flat-headed cats and fishing cats†

| Cell type | Stain | Clouded leopard | Flat-Headed Cat | Fishing Cat |
|---|---|---|---|---|
| Neutrophils | PER | + | ND | + |
| | SBB | + | + | + |
| | ANAE | - | - | +* |
| | β-glu | +* | - | + |
| | PAS | + | ND | + |
| Eosinophils | PER | - | ND | - |
| | SBB | - | - | - |
| | ANAE | - | - | +(intergranular) |
| | β-glu | - | +/-(intergranular) | +(peripheral granules) |
| | PAS | +(intergranular) | ND | + (intergranular) |
| Basophils | PER | - | ND | - |
| | SBB | - | - | - |
| | ANAE | + | + | + |
| | β-glu | + | + | + |
| | PAS | + | ND | + |
| Monocytes | PER | - | ND | +/- |
| | SBB | - | +/- | +/- |
| | ANAE | + | + | + |
| | β-glu | + | +/- | + |
| | PAS | + | ND | + |
| Lymphocytes | PER | - | ND | - |
| | SBB | - | - | - |
| | ANAE | + | + (fine granular) | + |
| | β-glu | + | + (focal) | + |
| | PAS | +/- (blocked) | ND | + |
| Platelets | PER | - | ND | - |
| | SBB | - | - | - |
| | ANAE | + | + | + |
| | β-glu | +/- | - | +* |
| | PAS | + | ND | +* |

† -, negative; +/-, positive or negative; +*, faintly positive, +, positive; PER, peroxidase; SBB, Sudan black B; ANAE, α-naphthyl acetate esterase; β-glu, β-glucuronidase; PAS, Periodic acid- Schiff; ND, not done

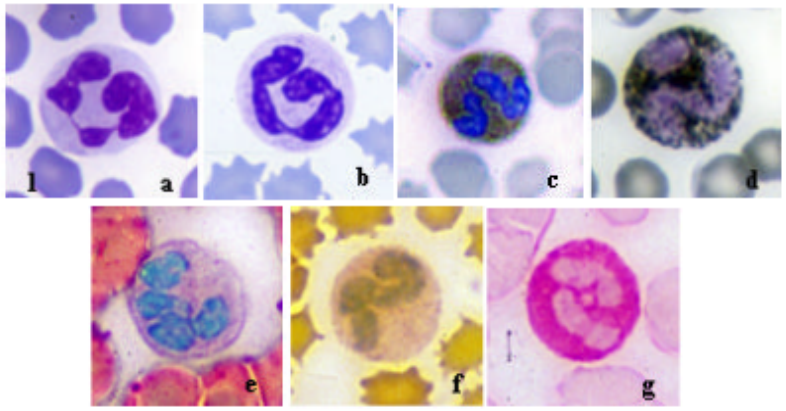


Fig. 1: Neutrophils: (a-b) a lobulated neucleus with indiscernible cytoplasmic granules in WG and W, respectively. (c) a strongly positive for PER. (d) a strongly positive for SBB. (e) a negatively stained for ANAE. (f) a weakly positive for β-glu. (g) a strongly positive with diffuse magenta stain, PAS

Ultrastructurally, neutrophil showed a lobulated nucleus with surface microvilli and numerous cytoplasmic granules (Fig. 2b-c).

**Eosinophils:** Eosinophil was 12.6 μm mean diameter (Table 2). They had segmented nuclei which were often bilobed. They had distinctive large round-shaped granules (Fig. 3a-b). Cytochemically, they were not stained with PER, SBB, ANAE and β-glu (Fig. 2c-f), while the refractive granules in magenta cytoplasmic background were seen in PAS (Fig. 2g). By SEM, they were globular shaped, depicting a large prominent granular contour (Fig. 2d). Ultrastructurally, they were round with lobed nucleus and poorly defined microvilli

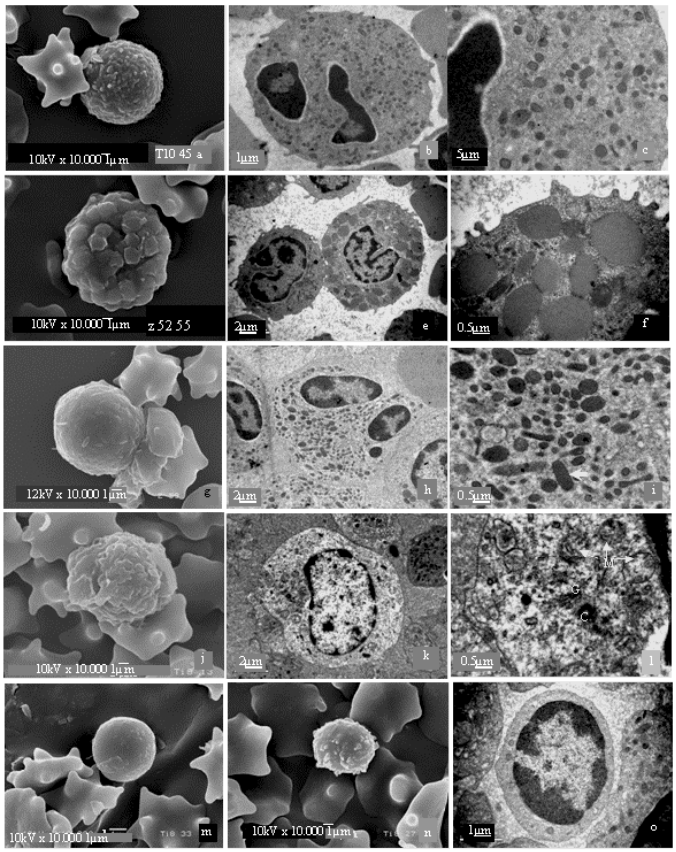

Fig. 2: Cellular surfaces and ultrastructures of various leukocytes in the clouded leopard. (a) a neutrophil presenting microvillous surface and micropores, SEM. (b) a neutrophil showing lobed nucleus with fine granules, TEM. (c) higher magnification of (b) presenting the granules, TEM. (d) an eosinophil showing large round prominent granular contour, SEM. (e) comparative cell types between neutrophil (left) and eosinophil (right), TEM. (f) higher magnification of (e) showing

(Fig. 2e; right). The cytoplasm contained characteristic large, round, electron dense granules ranging from 0.5-1μm in diameter (Fig. 2f).

**Basophils:** Basophils were found frequently on smears. The averaged size was 13.6 μm in diameter (Table 2). With WG, they had numerous fine blue-grey granules which did not obscure the lobed nucleus (Fig. 4a). The granules were not stained with W and appeared as refractive granules (Fig. 4b). For cytochemical profiles, they were negative for PER and SBB (Fig. 4c-d), but were positive for ANAE, β-glu and PAS appearing red-brown, bright red and magenta, fine granular patterns, respectively (Fig. 4e-g). By SEM, their surfaces revealed a low elevated pleomorphic granular contour, but rod granules were also prominent (Fig. 2g). Ultrastructurally, their cytoplasm was primarily occupied by heterogenous granules (Fig. 2h), which were occasionally detected with large rods (Fig. 2i).

**Monocytes:** Monocyte was 13.4 μm mean diameter (Table 2). The nuclei were extremely variable. They might be round, lobulated or band shape but usually deeply indented with lacy to reticular chromatin. The cytoplasm was blue-grey with occasionally variable sizes of vacuoles (Fig. 5a-b). They were negative for PER and SBB (Fig. 5c-d), but were moderately to strongly positive for ANAE, β-glu and PAS presenting in red-brown granular, bright-red granular and diffuse magenta patterns, respectively (Fig. 5e-g). With SEM,monocyte depicted irregular shapes with ruffle membrane (Fig. 2j). Ultrastructurally, the nuclei were of variable shapes with light cytoplasm containing several organelles which were seen easily (Fig. 2k-l).

**Lymphocytes:** Lymphocytes varied from 8.3-15.1 μm in diameters. The average sizes of small, medium and large lymphocytes were 8.6, 10.8 and 14.4 μm, respectively (Table 2). Most lymphocytes were small and medium

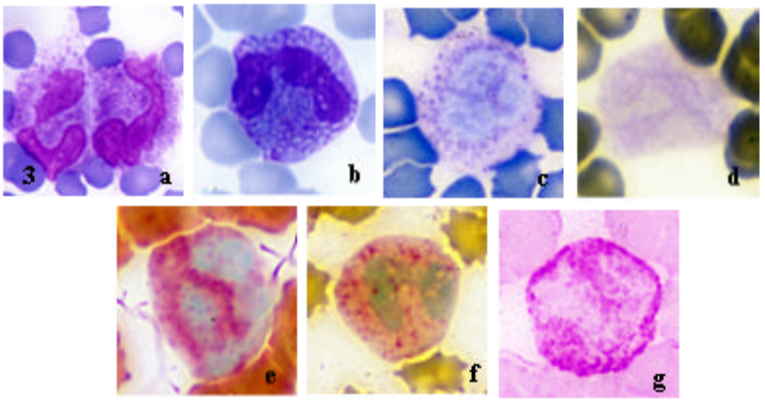


Fig. 3: Basophils: (a) 2 basophils with large lobed nucleus and fine blue-grey granules, WG. (b) a large lobed nucleus with non-stained granules, W. (c) negatively stained for PER. (d) negatively stained for SBB. (e) moderately positive for ANAE. (f) moderately positive for β-glu with granular pattern. (g) positively stained for PAS with fine granular pattern

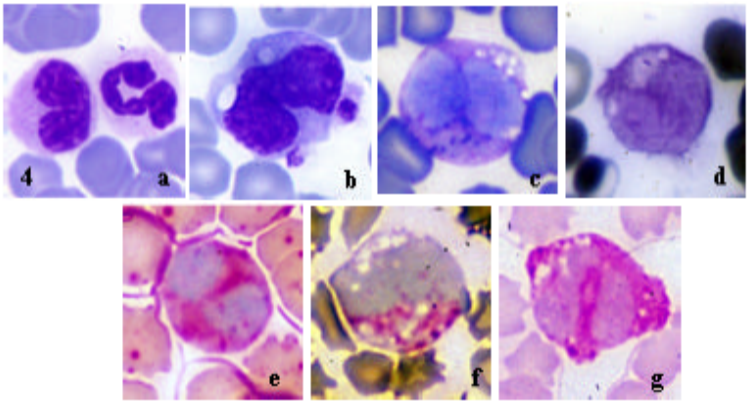


Fig. 4: Monocytes: (a) comparative nucleus of monocyte (left) to neutrophil (right), WG. (b) a monocyte with deep indented nucleus and vacuolated cytoplasm, W. (c) negatively stained for PER. (d) negatively stained for SBB. (e) strongely positive for ANAE. (f) positively stained for β-glu with granular pattern. (g) positively stained for PAS with diffusely pattern

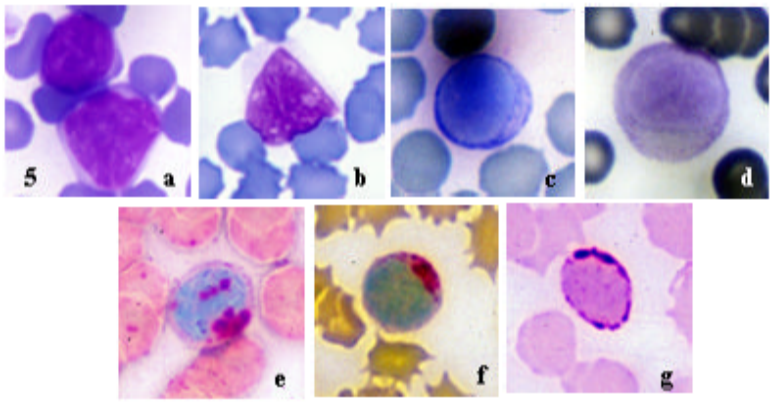


Fig. 5: Lymphocytes: (a) two lymphocytes, WG. (b) a lymphocyte with dense nucleus and thin band cytoplasm, W. (c) negatively stained for PER. (d) negatively stained for SBB. (e) positively stained for ANAE with coarse granular pattern. (f) positively stained for β-glu with red focal granules. (g) positively stained for PAS with block-liked pattern

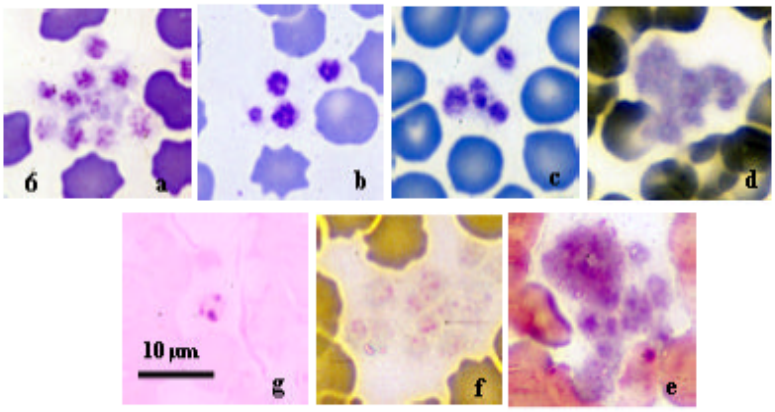


Fig. 6: Platelets: (a-b). group of small round, anucleated cells with reddish-purple granules, WG and W, respectively. (c) negatively stained for PER. (d) negatively stained for SBB. (e) moderately positive for ANAE. (f) weakly positive for β-glu. (g) moderately positive for PAS

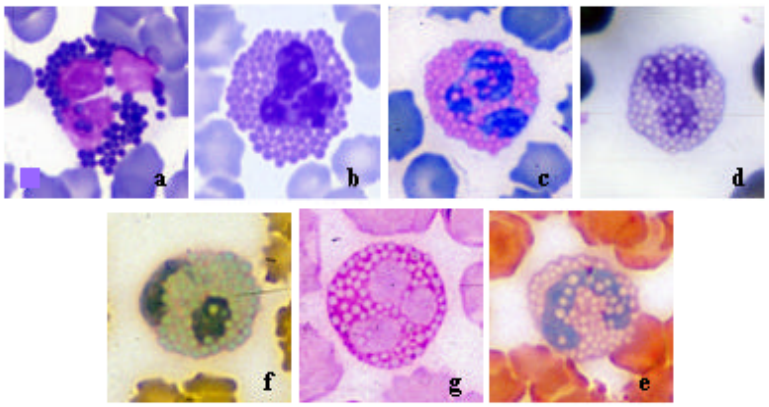


Fig. 7: Eosinophils: (a-b) lobulated neucleus with large prominent cytoplasmic granules in WG and W, respectively. (c) reddish refractive granules in negatively stained for PER. (d) refractive granules in negatively stained for SBB. (e) refractive granules in negatively stained for ANAE. (f) refractive granules in negatively stained for β-glu. (g) refractive granules in positively stained cytoplasmic background, PAS

sizes (Fig. 6a-b). They were negative for PER and SBB (Fig. 6c-d), but had 2 patterns of reactivity with ANAE and β-glu, including coarse granular and focal granular patterns (Fig. 6e-f). Most lymphocytes were negative for PAS, but a positive block-liked pattern was found in some lymphocytes (Fig. 6g). By SEM, they were bulgy with membrane blebs (Fig. 2m) or small membrane projections (Fig. 2n). Ultrastructurally, their nuclei were generally round or oval occupying the greater part of the cells (Fig. 2o).

**Platelets:** Platelets were approximately one-fifth to one-half of red blood cells with reddish-purple granules easily seen in WG and W (Fig. 7a-b). Cytochemically, they were negative for PER and SBB (Fig. 6c-d), weakly positive for β-glu (Fig. 7f) and moderately positive for ANAE and PAS (Fig. 7e-g).

## DISCUSSION

Morphological feature of red blood cells in the clouded leopard were generally similar to those of domestic and some wild cats; fishing cat and flat-headed cat. By SEM, the microvillous surfaces of neutrophils and small membrane projections of lymphocytes probably played a significant role in the adhesion to vascular endothelium against the force of blood flow and the irregular shapes of monocytes might relate to the behavior

of the cells, because they were able to become macrophages which often extended processes such as pseudopodia (Steffens, 2000), while the blebs of lymphocytes were unrelated to the functional identity (Jain, 1993). Eosinophils were more easily identified than other granulocytes because of their distinctive large granules. This characteristic was different from rod shaped granules of eosinophils of the domestic cat, fishing cat and flat-headed cat (Reagan, *et al*., 1998; Prihirunkit *et al*., 2007; Salakij *et al*., 2007). Unlike eosinophils of most species, eosinophils of the clouded leopard were stained with neither PER nor SBB, but these findings were the same as eosinophils of the domestic cat (Tsujimoto *et al*., 1983), the fishing cat (Prihirunkit *et al*., 2007) and the flat-headed cat (Salakij *et al*., 2007). The negative for ANAE and β-glu, but strongly positive for PAS in cytoplasmic background indicated that neither α-naphthylacetate esterase nor β-glucuronidase was contained in eosinophil, while a large amount of glycogen was (Hayhoe and Quaglino, 1980).

In WG, prominent fine blue-grey granules of basophils were similar to those of the flat-headed cat (Salakij *et al*., 2007), but were different from those of the domestic cat and fishing cat which were lavender stained cytoplasm (Reagan, *et al*., 1998; Prihirunkit *et al*., 2007). The cytochemical profiles of leukocytes except for the eosinophil of clouded leopard were similar to those of the fishing cat (Prihirunkit *et al*., 2007).

## CONCLUSION

The prominent morphologies of blood cells in the clouded leopard were eosinophils and basophils which were characteristically large round and fine blue-grey granules, respectively. Cytochemistry provided the staining patterns among the cell types. The electron microscope was beneficial in the identification of cellular surfaces as well as cytoplasmic granules and organelles. This information provided a guideline for the identification of blood cells in the clouded leopard.

## ACKNOWLEDGMENT

The authors gratefully acknowledge to Kasetsart University Research and Development Institute for funding the study. Deep appreciation is expressed to Ms Piyawan Suthunmapinuntra and Ms Nirachara Rochanapat for their assistance in fieldwork.

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