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Hematology, Cytochemistry and Ultrastructure of Blood Cells in Asiatic Black Bear (*Ursus thibetanus*)

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ABSTRACT

Blood cells from adult Asiatic black bear *(Ursus thibetanus)* were examined and measured after stainning with modified Wright stain and cytochemical stains, including Sudan Black B (SBB), Periodic acid Schiff's reaction (PAS), a-naphthyl acetate esterase (ANAE), acid phospatase (AcP) and b-glucuronidase (b-glu). Red blood cells were uniform in shape, with 7.3 mm mean diameter in size and easy to form rouleaux. Using scanning electron microscopic (SEM) examinations revealed normal and abnormal red blood cell surfaces. Neutrophils contained several vacuoles as detected by light microscope and revealed themselves as large granules under transmission electron microscope. Neutrophils stained strongly positive with SBB, ANAE; weak positive with PAS and negative with AcP and b-glu. Using SEM, neutrophil surfaces revealed several microvilli and some micropores. Eosinophils contained numerous small round red refractive granules with some vacuoles. Eosinophils stained strongly positive with SBB and ANAE but negative with PAS and b-glu. Basophils had variable numbers of intense basophilic granules that obscured the very long lobulated nucleus. Basophils stained moderately positive with SBB but strongly positive with ANAE. Lymphocytes were negative with SBB but have 3 patterns of reactivity with ANAE, AcP and b-glu, including negative, focal dot and fine granular stainings. Monocytes stained moderately with SBB and moderately to strongly with for ANAE and b-glu. The SEM examinations could differentiate white blood cells by their surface contours. Transmission electron microscopic examinations revealed organelles within all blood cells.

Key words: Asiatic black bear, blood cell, cytochemistry, morphology, ultrastucture

INTRODUCTION

Asiatic black bear (*Ursus thibetanus*) has shaggy black fur with white crescent on the chest, considerably larger than Malayan sun bear (*Ursus malayanus*). Asiatic black bear has suffered from habitat loss and is now rare in many areas (Francis, 2001). So this endangered species has been studied intensively to determine the health status of the individuals. Veterinary hematology serves as a screening procedure to assess general health, the body's ability to fight infection in adjunct to patient evaluation or diagnosis (Jain, 1993).

Differential white blood cell count is very useful not only in numbering the white blood cells but also provide evidence of anemic condition or

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reveal the pathogenesis. Blood smear examinations provide more information on morphology of red blood cell, white blood cell and platelets (Mills, 1998). Cytochemical method is useful in diagnosis of acute leukemia in human (Apibal, 1987; Khemtonglang *et al*., 1997). The purpose of the present study was to characterize the morphology, cytochemical reaction and ultrastrucrure of blood cells in Asiatic black bear.

MATERIALS AND METHODS

From February to June 2003, five clinically healthy Asiatic black bears in Khao Kheaw Open Zoo were chemical restrained with xylaxine (Rompun®) and ketamine. Two millilitres of blood samples were collected from the jugular vein and transferred to tubes containing ethylenediamine tetraacetic acid (EDTA). Some of the blood without anticoagulant was directly smeared on the slides. Two Asiatic black bears were adult males and three were adult females aging between 3-5 years old. Hematology, plasma protein and fibrinogen were determined by manual technique (Schalm *et al.*, 1975) within two hours after blood collection. Two direct blood smears from each bear were stained with a modified Wright and Wright's stains. A minimum of 200 leukocytes were counted for differential leukocyte determinations. The anticoagulated blood was used for reticulocyte count by staining with new methylene blue stain (Schalm *et al.*, 1975). The percentage of reticulocyte presented in 1,000 red blood cells (RBC) was determined. For each hematologic parameter, means, variances and standard errors were calculated using SPSS® for Window™ (Norusis, 1993).

Cytochemical staining characteristics of blood cells were evaluated using air-dried blood smears from three Asiatic black bears. Cells were stained with periodic acid Schiff's reaction (PAS), Sudan black B (SBB), a–naphthyl acetate esterase (ANAE), acid phosphatase (AcP) and b–glucuronidase (b–glu). Cytochemical procedures used were the same as those previously described (Salakij *et al*., 2002). Positive– and negative-stained cells were differentiated by counting 500 cells on each of the cytochemically stained smears.

For scanning electron microscopy (SEM) and transmission electron microscopy (TEM), blood cells from three Asiatic black bears were processed as described by Salakij *et al.* (2002). Identification of blood cells by SEM and TEM was based on the relative number, size, shape and distribution of granules and on nuclear appearance.

RESULTS

There was no blood parasite detected in all bears. Hematological data of Asiatic black bear was tabulated (Table 1). White blood cell differential counts were shown in Table 2. Blood cell diameters were observed and calculated (Table 3). Cytochemical staining patterns of blood cells were summarized (Table 4). The morphology under light microscope, SEM, TEM and cytochemical characteristics of individual blood cells were evaluated, as described below.

Erythrocytes

Red blood cells (RBCs) or erythrocytes under light microscope showed uniform in shape (Figure 1), slightly biconcave and slightly central pallor as observed by SEM (Figure 4a, 4b) with 7.3 mm mean diameter in size (Table 3) and easy to forming rouleaux. Some defected RBCs (Figure 4c), crenated RBCs (Figure 4d), echinocyte (Figure 5a, 5b), rubricyte (Figure 8b) and metarubricyte (Figure 8c) were also observed. Mature red blood cells were negative for all cytochemical stainings. Ultrastructurally, mature RBC showed only hemoglobin (Figure 8a) while the metarubricytes showed some organelles (Figure 8c) and the rubricytes showed more mitochondria (Figure 8b).

Table 1 Hematology of Asiatic black bear.

Hematology	Asiatic black bear (n = 5)
PCV (%)	41.4 ± 1.8
Hemoglobin (g/dL)	13.3 ± 0.6
RBC (10^{12}/L)	6.08 ± 0.39
MCV (fL)	68.5 ± 2.1
MCHC (g/dL)	32.1 ± 0.6
WBC (10^{11}/L)	6.99 ± 0.59
Band neutrophils (10^9/L)	0.11 ± 0.09
Segmented neutrophils (10^9/L)	4.15 ± 0.19
Lymphocytes (10^9/L)	1.84 ± 0.28
Monocytes (10^9/L)	0.13 ± 0.04
Eosinophils (10^9/L)	0.73 ± 0.18
Basophils (10^9/L)	0.03 ± 0.02
Band neutrophils (%)	1.7 ± 1.4
Segmented neutrophils (%)	60.3 ± 3.4
Lymphocytes (%)	25.8 ± 1.7
Monocytes (%)	1.8 ± 0.5
Eosinophils (%)	10.0 ± 1.5
Basophils (%)	0.4 ± 0.2
Plasma protein (g/dL)	8.1 ± 0.3
Fibrinogen (mg/dL)	220 ± 49
Reticulocyte (%)	0.0 ± 0.0

Platelets

Bear platelets were approximately 1/5 to 1/2 of RBC and had prominent reddish-purple granules which were easily seen in modified Wright stain (Figure 1d, f). Plateletes were not stained with SBB but were moderately to strongly positive with ANAE (Figure 3m). These platelets were seldom seen on RBCs (Figure 5b), but gave rosette formation on monocyte (Figure 5c) and aggregation (Figure 5d). Ultrastructurally, platelets showed dense granules, alpha-granules, glycogen granules and microtubule (Figure 8d).

Table 2 White blood cell differential count in Asiatic black bear.

Hematology	Asiatic black bear (n = 5)
Band neutrophils (%)	1.7 ± 1.4
Segmented neutrophils (%)	60.3 ± 3.4
Lymphocytes (%)	25.8 ± 1.7
Monocytes (%)	1.8 ± 0.5
Eosinophils (%)	10.0 ± 1.5
Basophils (%)	0.4 ± 0.2

Neutrophils

Neutrophils were the most prevalent leukocyte in Asiatic black bear (Table 2) with neutrophil : lymphocyte ratio equal to 62 : 26. With modified Wright stain, neutrophils showed the same size as basophil (Table 3). Neutrophils showed faintly stained cytoplasm which contained indistinct pale granules and several vacuoles

Table 3 Mean ± SD of blood cell diameters (μm) in Asiatic black bear.

Cell type	No.	Asiatic black bear
Red blood cells	50	7.30 ± 0.84
Segmented Neutrophils	50	13.42 ± 1.10
Eosinophils	50	14.09 ± 1.66
Basophils	18	13.06 ± 1.95
Lymphocytes		
small	50	8.28 ± 0.78
medium	50	11.02 ± 1.11
large	17	14.40 ± 0.48
Monocytes	50	15.28 ± 2.14

Table 4 Cytochemical staining patterns of blood cells from Asiatic black bear.

Cell type	SBB	PAS	ANAE	Acid phosphatase	b-glucuronidase
Neutrophils	+ +	+	++	-	±±
Eosinophils	+	-	+++	-	+
Basophils	+ +	-	++	-	-
Lymphocytes	-	-	- / focal dot / fine granular	- / focal dot / fine granular	- / focal dot / fine granular
Monocytes	+	-	+ +	-	+
Platelets	-	-	++	-	-

SBB indicates sudan black B; PAS, periodic acid-Schiff; and ANAE, a-naphthyl acetate esterase. Staining was scored as negative (-), weak (±±, few positive cells), moderate (+), moderate to strong (++), or strong (+++).

(Figure 1a, 2a). Neutrophils have tight constricted and multilobulated nuclei (Figure 1a, 2a). These vacuoles shown in light microscope were revealed as large granules in TEM (Figure 9a-d). Some neutrophils (2-5%) of the female revealed sex chromatin lobe.

Neutrophils stained strongly positive with SBB (Figure 3a), moderately stained with ANAE (Figure 3i) and negative with b-glu (Figure 3o). Using SEM, neutrophil surfaces revealed several microvilli and some micropores (Figure 6a, 6b, 6d). Ultrastructurally, neutrophils showed lobed nuclei, small specific granules (Figure 11a, b), large electron-dense granules (Figure 9a-c) and glycogen granules (Figure 9d) were also detected.

Eosinophils

Eosinophils varied from 10 to 16 μm (average 14 μm) in diameter (Table 3). Eosinophils contained numerous small round red refractive granules with some vacuoles (Figure 1b, 2b). Eosinopil nuclei were less lobulated than those of neutrophils, and tetralobed, trilobed or band-shaped. Eosinophils stained moderately positive with SBB (Figure 3b) and ANAE (Figure 3j) but weak positive with b-glu (Table 4). Under SEM, eosinophil surfaces revealed larger granule contour (Figure 6c, 6d) than those of basophils (Figure 6e, 6f). Ultrastructurally, eosinophils showed lobed nuclei, large pleomorphic granules with bar-shape structures in some granules, Golgi apparatus, RER and ribosomes (Figure 10a, 10b).

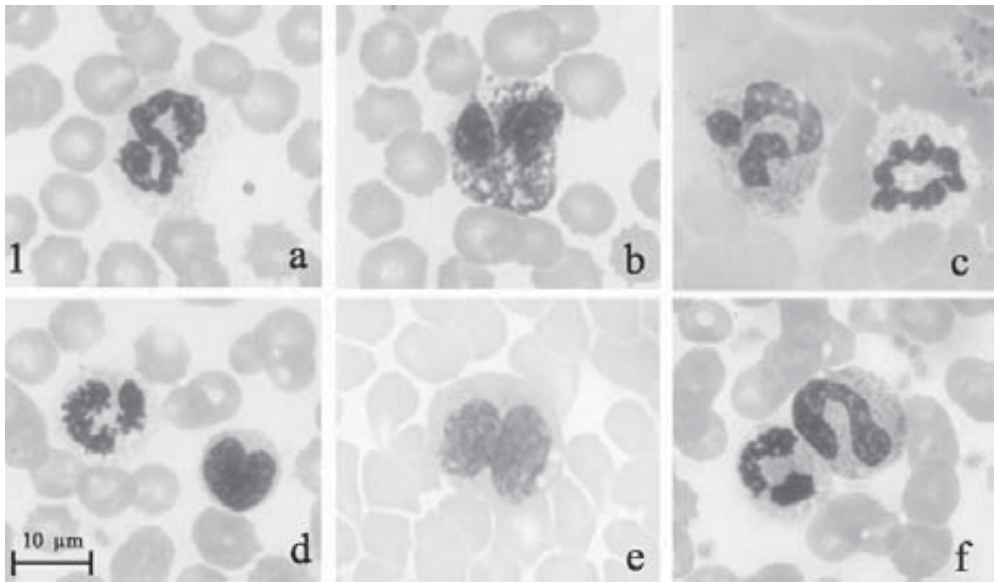

Figure 1 Light micrographs of blood cells in Asiatic black bear stained with modified Wright stain. a. A segmented neutrophil with many cytoplasmic vacuoles. b. An eosinophil. c. A basophil (left) and a segmented neutrophil. d. A lymphocyte (right) and a segmented neutrophil. e. A monocyte. f. A basophil (right) and a segmented neutrophil.

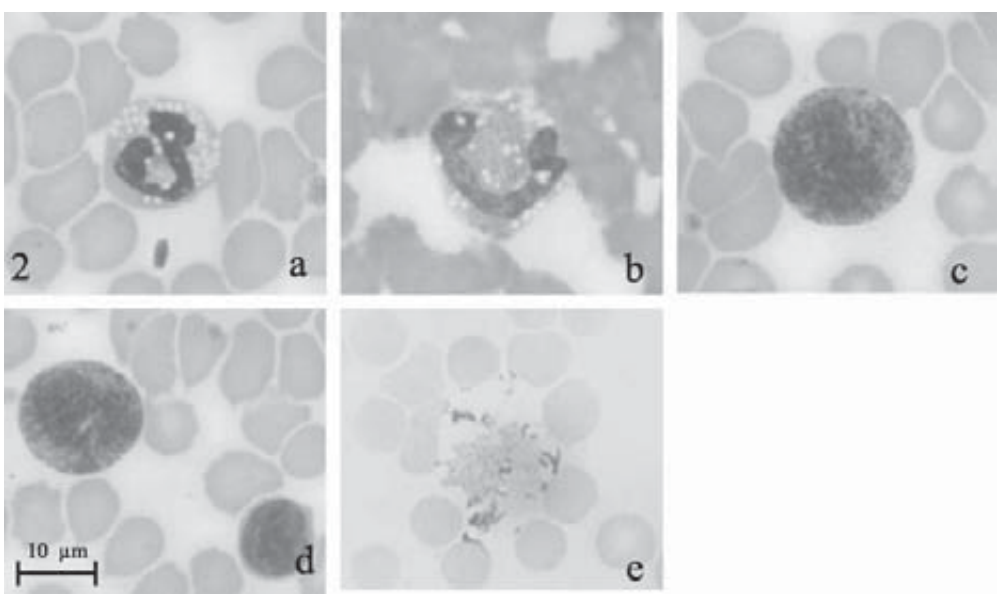

Figure 2 Light micrographs of blood cells in Asiatic black bear stained with Wright's stain (a-d). a. A segmented neutrophil with some cytoplasmic vacuoles. b. An eosinophil. c. A basophil. d. A lymphocyte (right) and a basophil. e. A leukocyte stained with new methylene blue whilst there was no reticulocyte.

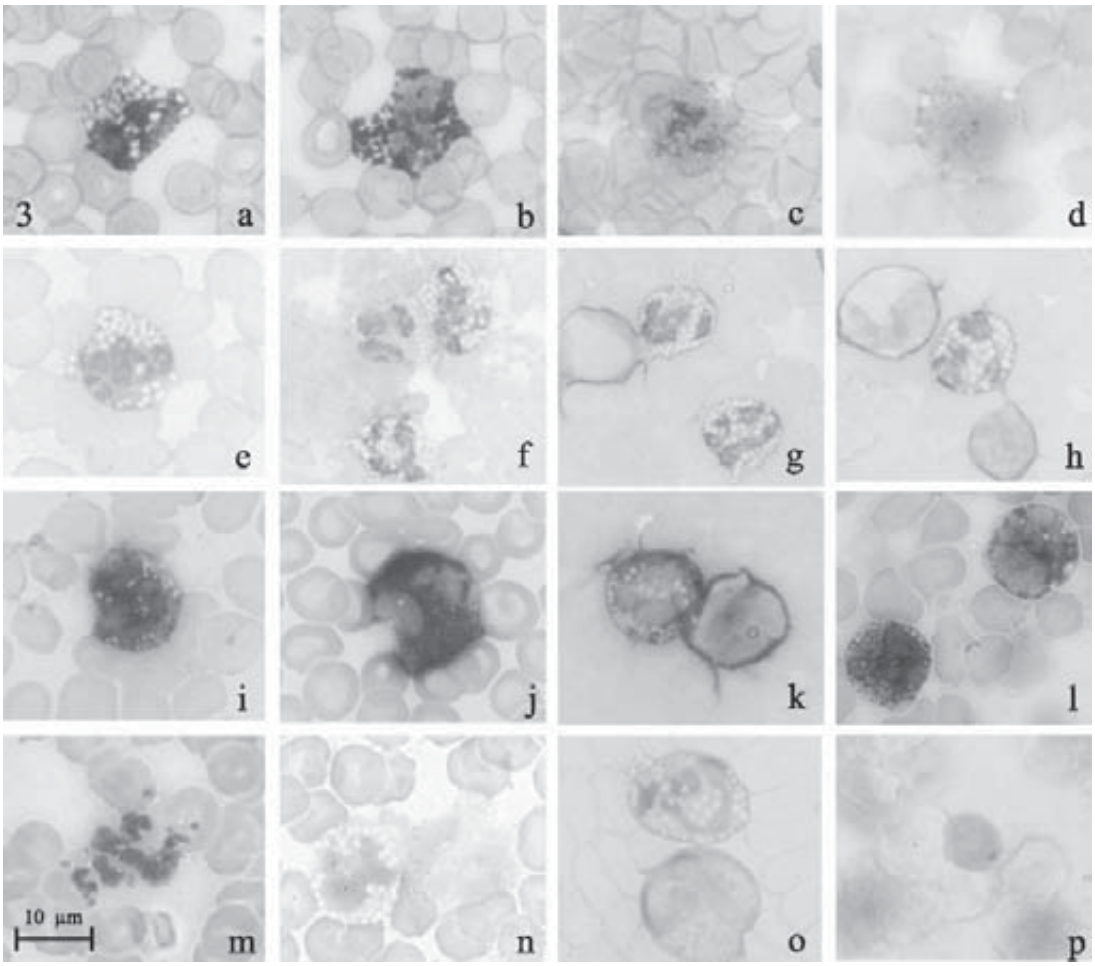

Figure 3 Cytochemical staining of blood cells in Asiatic black bear a. Sudan black-B (SBB) positive segmented neutrophil. b. SBB positive in the periphery of the granules of the eosinophil. c. SBB positive in basophil. d. Some small dots of SBB positive in a 14 mm monocyte. e. PAS positive in segmented neutrophil. f. PAS positive in two segmented neutrophils and negative in an eosinophil. g. PAS negative in a basophil comparing with PAS positive in two segmented neutrophils. h. PAS negative in lymphocyte (lower right) and monocyte (upper left). i.-m. ANAE reactivity in a segmented neutrophil (i), eosinophil (j), basophil (right) (k), monocyte (upper right) (l) and in platelets (m). n. Negative AcP in a segmented neutrophil and a monocyte. o. Negative b-glu in a segmented neutrophil and a basophil. p. Focal dot positive of b-glu in a lymphocyte.

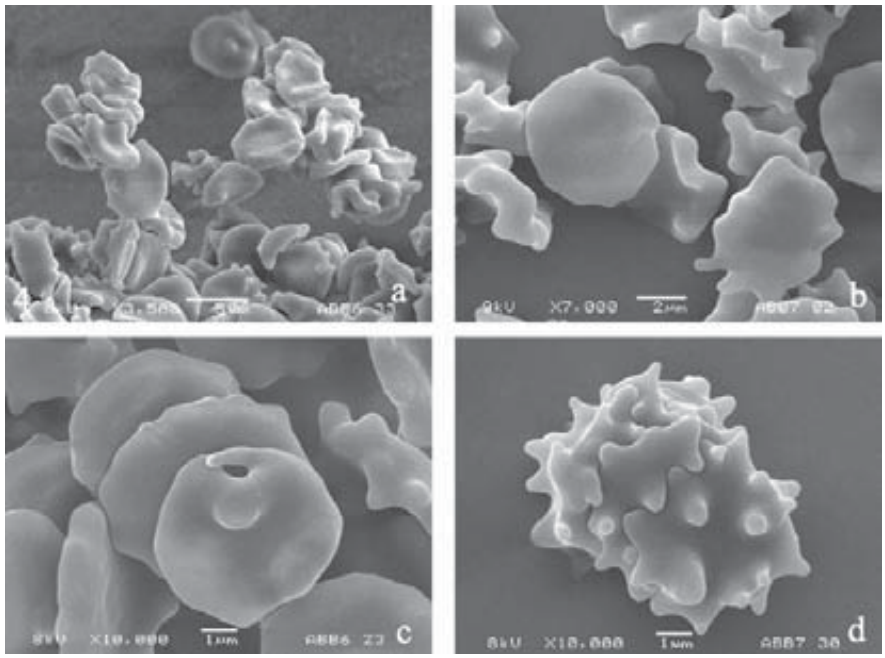

Figure 4 Scanning electron micrographs (SEM) of red blood cells (RBCs) in Asiatic black bear. a. A cluster of RBCs showing bicocave disk and crenations. b. Higer magnification of crenated RBCs. c. Defective RBCs. d. A cluster of four echinocytes.

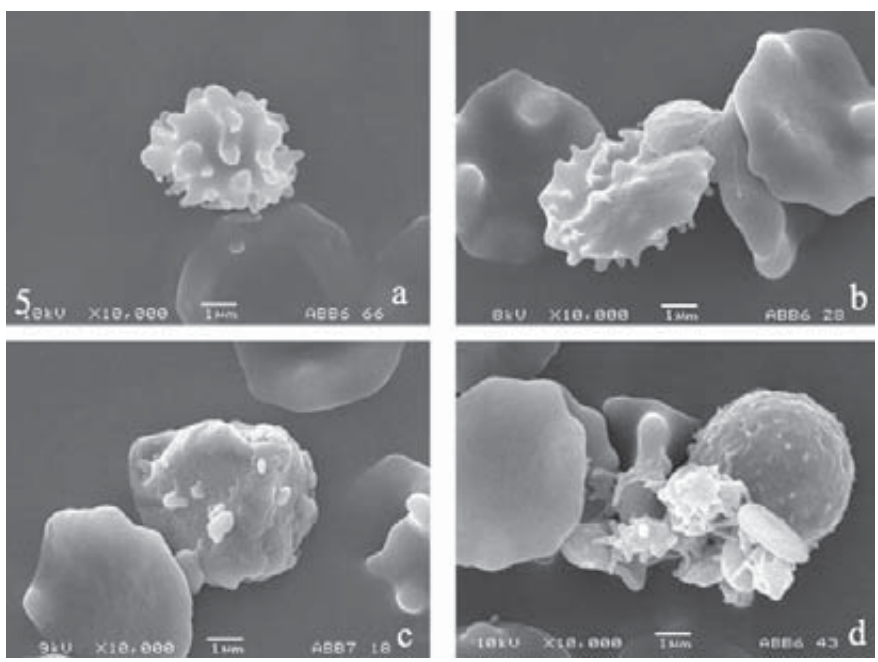

Figure 5 SEM of RBCs and platelets in Asiatic black bear. a. An echinocyte. b. A platelet on an echinocyte. c. Plateletes rosetting on a monocyte. d. Platelets aggregation next to neutrophil.

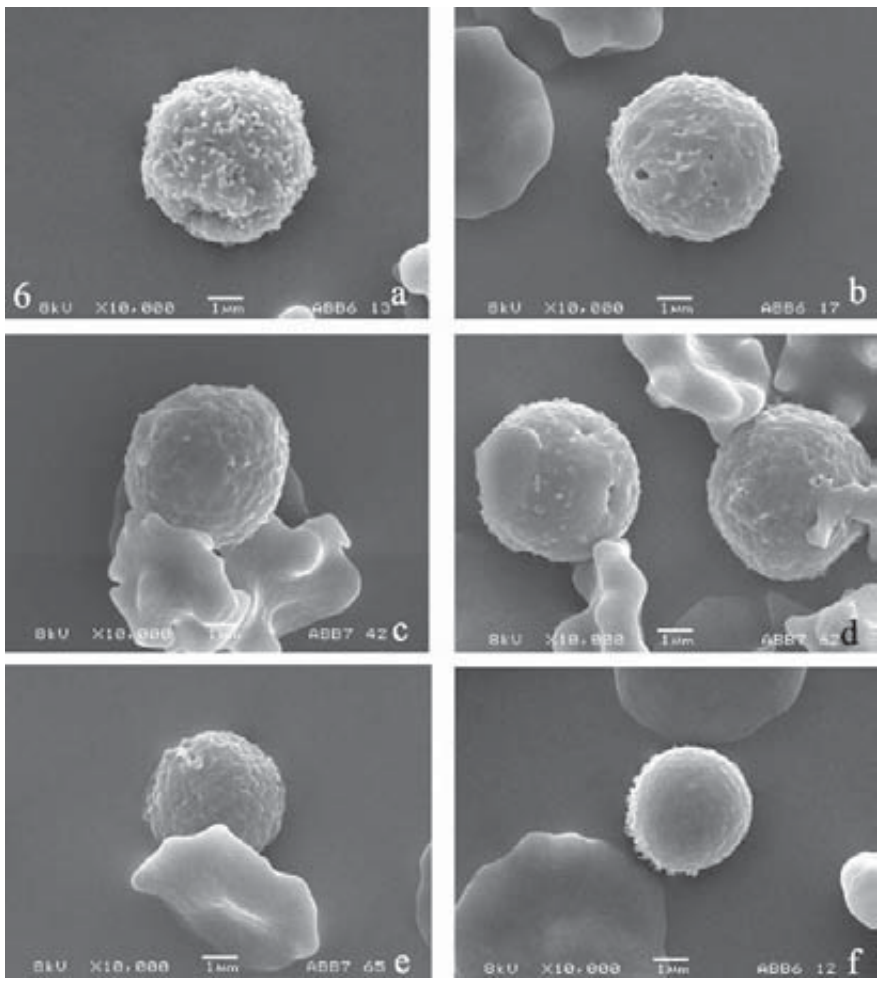

Figure 6 SEM of granulocytels in Asiatic black bear. a. A neutrophil showing short microvilli and some micropores. b. A neutrophil with three micropores. c. An eosinophil. d. An eosinophil (right) and a monocyte (left). e. A basophil showing small granule contour. f. A basophil.

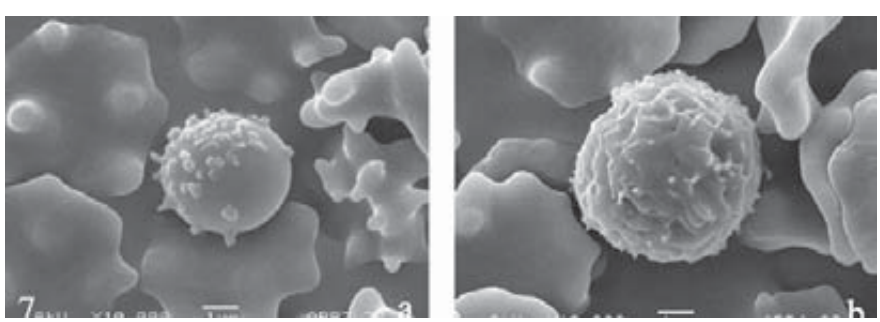

Figure 7 SEM of agranulocytes in Asiatic black bear. a. A lymphocyte with several cytoplasmic blebs. b. A monocyte with deep surface fissures.

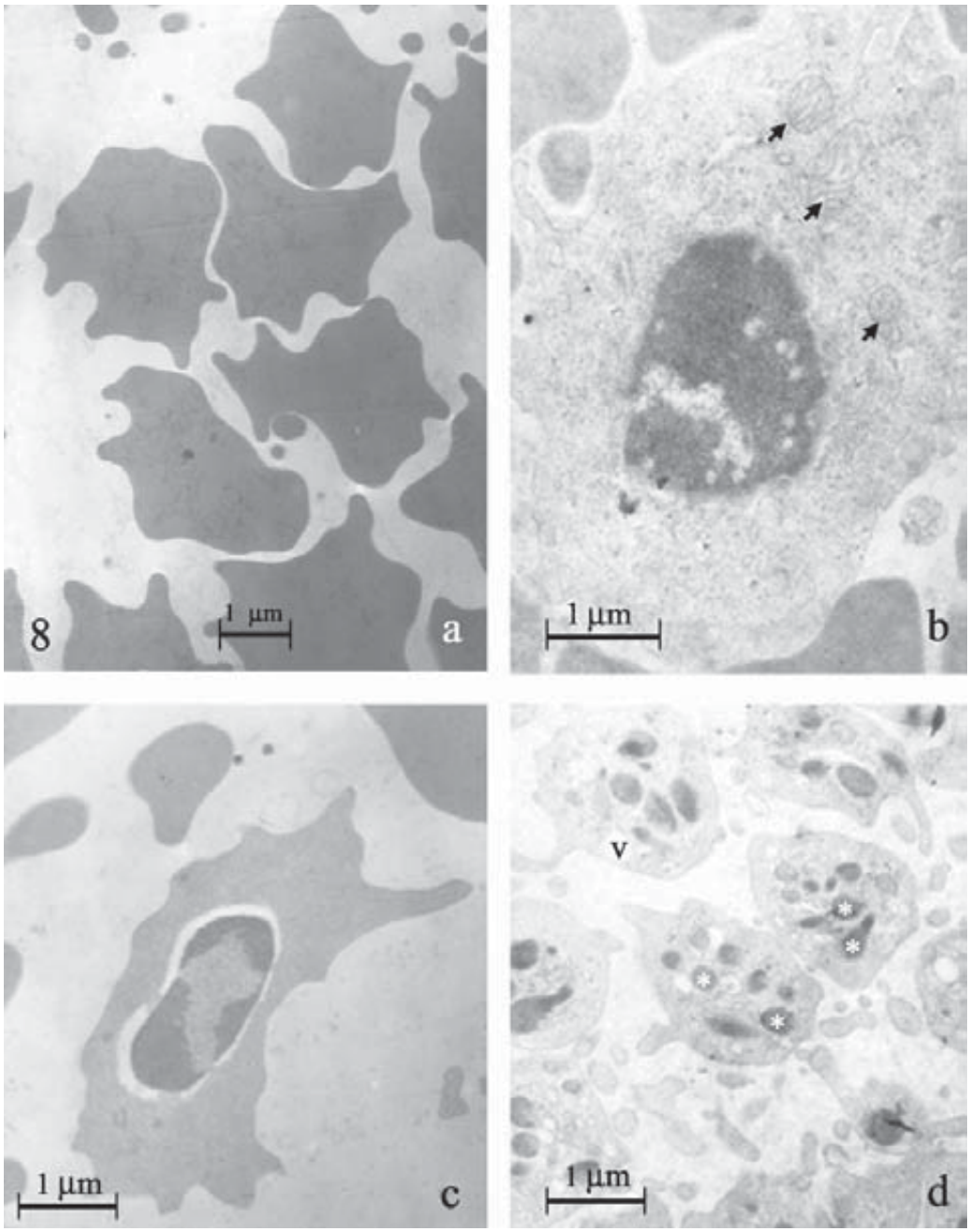

Figure 8 Transmission electron micrographs (TEM) of blood cells in Asiatic black bear. a. Mature erythrocytes. b. A rubricyte with some mitochondria (arrows). c. A metarubricyte. d. Platelets containing mitochondria, vacuoles (v) and dense granules (*).

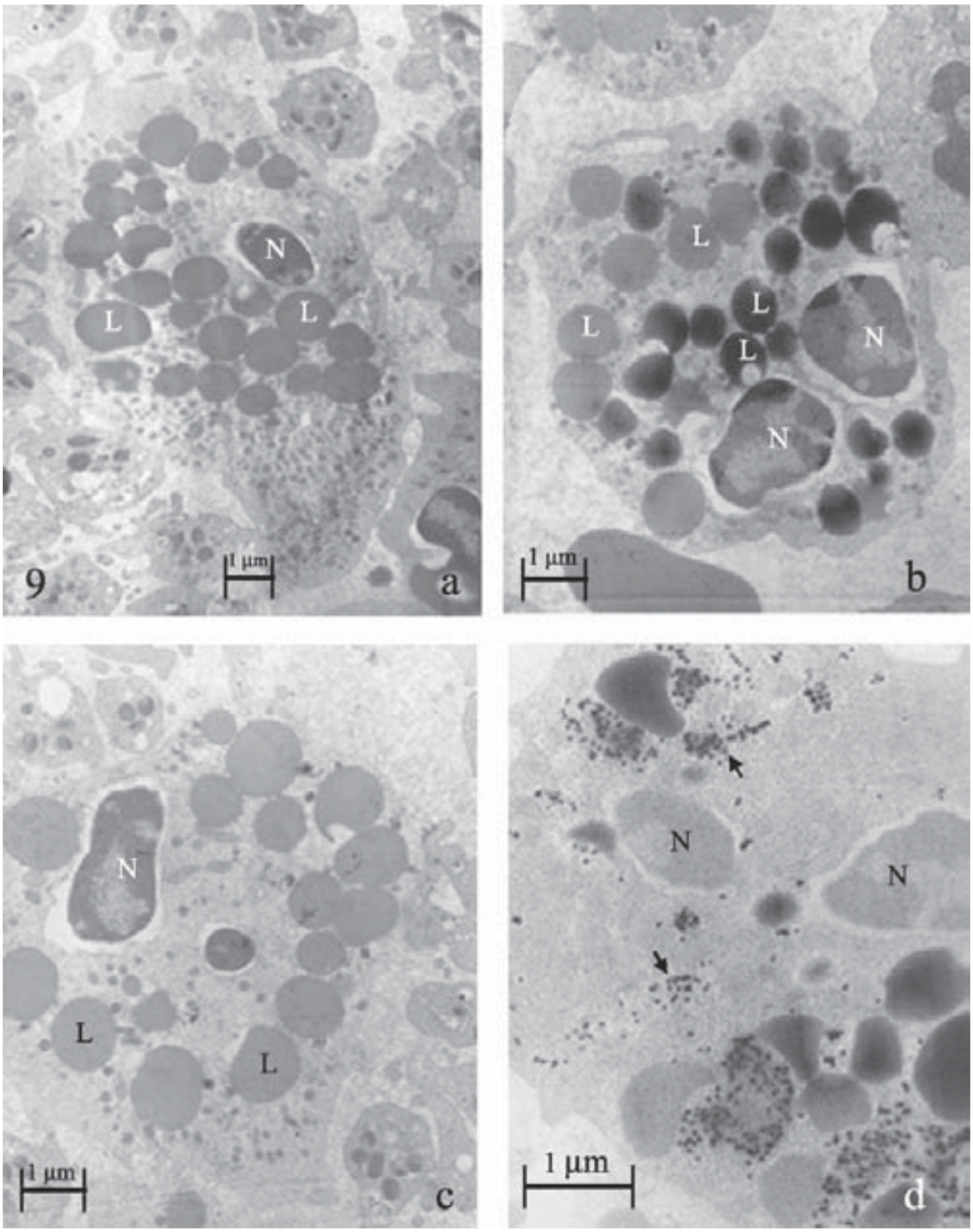

Figure 9 TEM of neutrophils in Asiatic black bear. a. A segmented neutrophil showing nucleus (N) many fine granules and large granules (L). b. A segmented neutrophil showing two lobed neucleus (N) with heterogeneous granule density of large granules (L). c. A segmented neutrophil (N) with homogeneous granule density of large granules (L). d. A segmented neutrophil (N) with many glycogen granules (arrows).

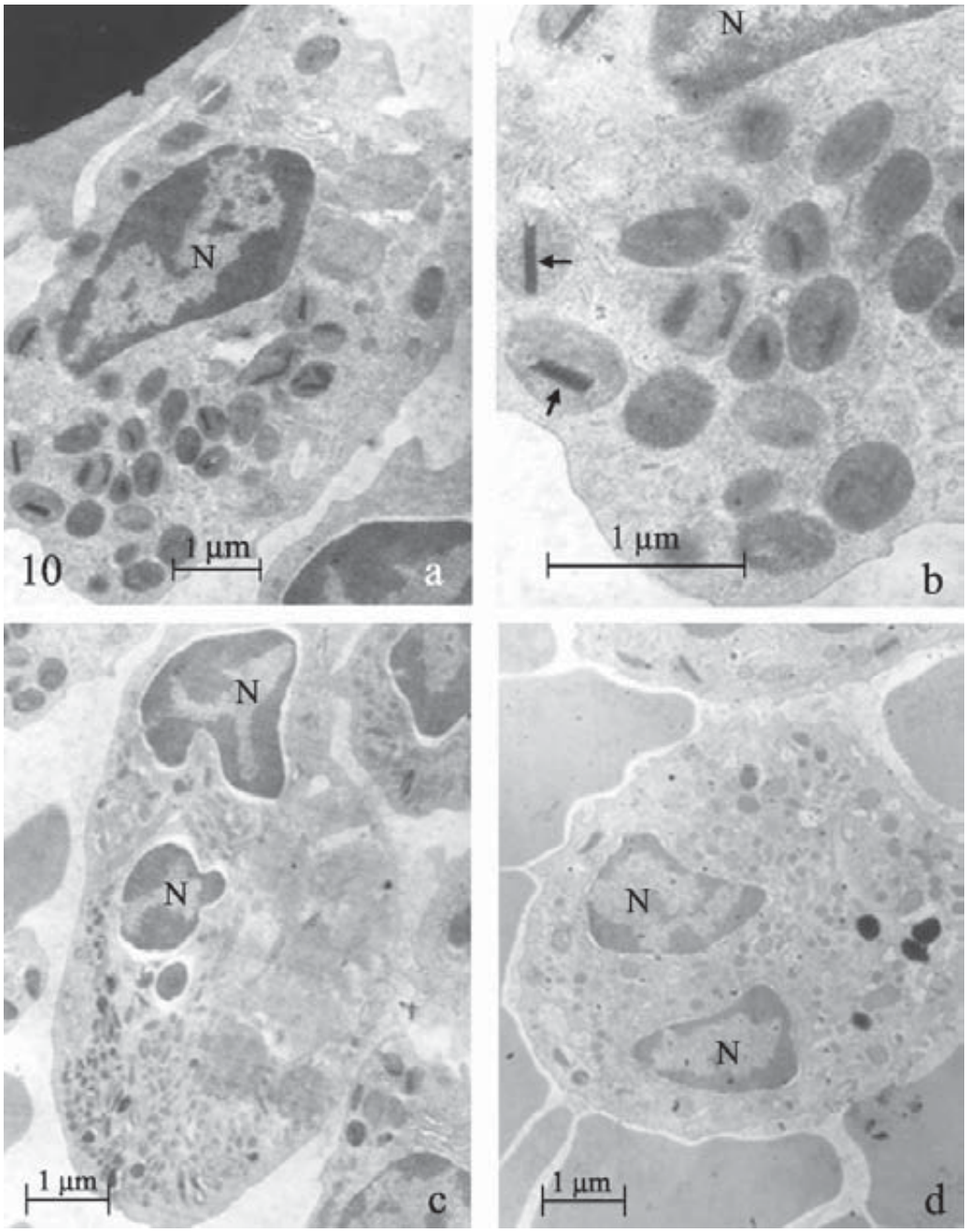

Figure 10 TEM of eosinophils and basophils in Asiatic black bear. a. An eosinophil showing neucleus (N) and many granules. b. Higher magnification of eosinophil in (a) showing bar-shape structures (arrows) in their granules. c., d. Basophils showing bilobed neuclei (N) and many small heterogenous granules.

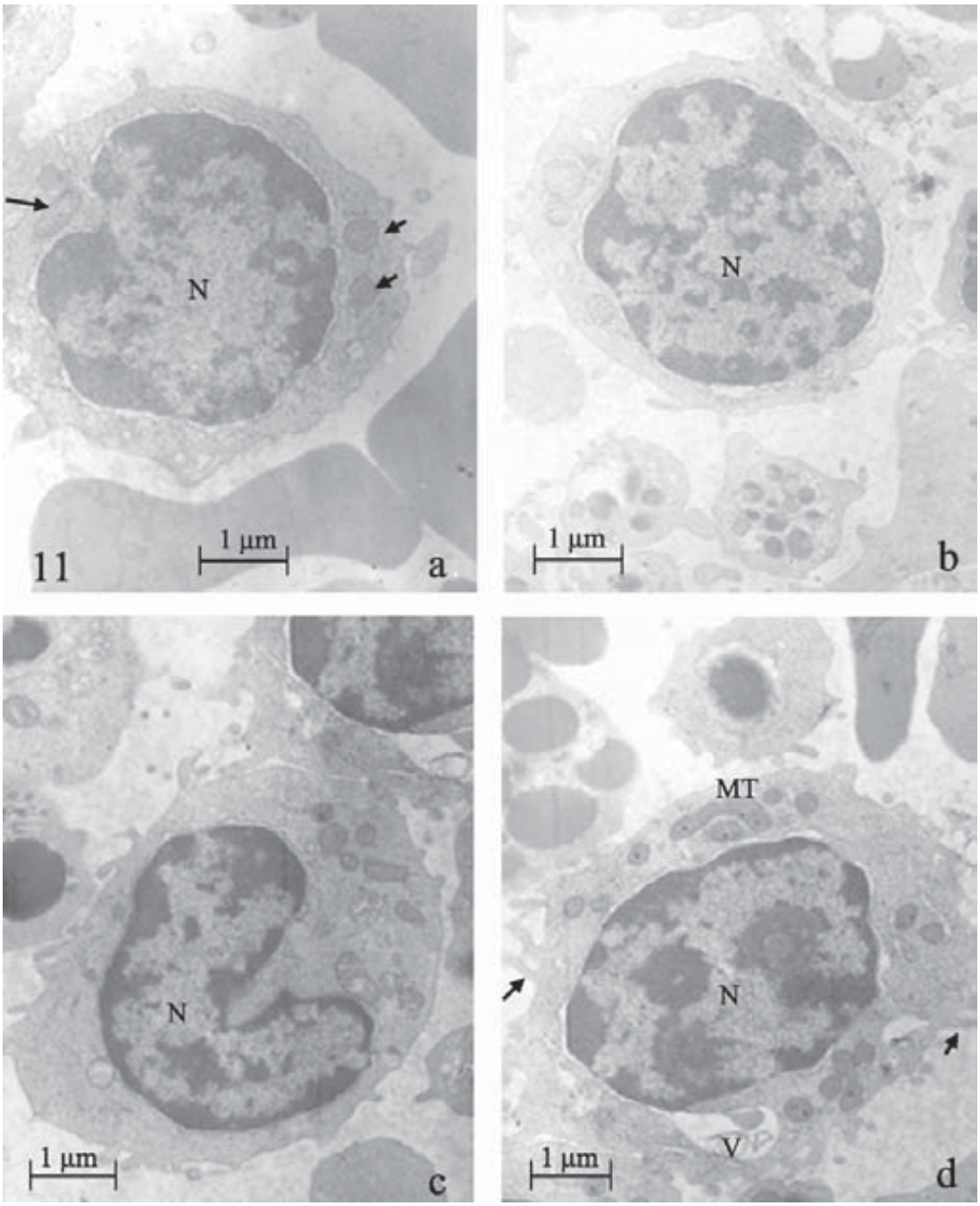

Figure 11 TEM of lymphocytes and monocytes in Asiatic black bear. a. A lymphocyte showing round neucleus (N) and three mitochondria (arrows). b. A lymphocyte with round nucleus (N). c. A monocyte with kidney-shaped nucleus (N). d. A monocyte with nucleus (N), many mitochondria (MT), ribosomes, vacuoles (v) and pseudopodia (arrows).

Basophils

Basophils in were not frequently observed. They varied from 11 to 15 mm (average 13 mm). Basophils contained variable numbers of intensely basophilic granules (Figure 1c, 1f, 2c, 2d) which obscured the very long lobe nucleus. Basophil granules stained with SBB more faintly than granules of neutrophil (Figure 3c). Basophil granules appeared red-brown when stained with ANAE (Figure 3k) but weak positive or negative with b-glu (Figure 3o). Under SEM, basophil surfaces revealed smaller granule contour (Figure 6e, 6f) than those of eosinophils (Figure 6c, 6d). Ultrastructurally, basophils showed lobed nuclei, small heterogenous electron density granules, some dense granules (Figure 10c) and some mitochondria (Figure 10d).

Lymphocytes

Lymphocytes in bears were variable in size (7 to 16 mm diameter). Most lymphocytes were small (Figure 1d, 2d) and medium size. Some lymphocytes contained small azurophilic granules in their cytoplasm. Lymphocytes were negative for SBB but had 3 patterns of reactivity for ANAE and b-glu, including negative, focal dot and fine granular stainings. Under SEM, lymphocyte surfaces revealed smooth bulging contour of round nuclei (Figure 7a) with variable numbers of cell membrane blebs. Ultrastructurally, lymphocytes showed round nuclei with peripheral clumps of heterochromatin and some mitochodria (Figure 11a, 11b).

Monocytes

Monocytes in bears varied from 13 to 17 mm in diameter (Table 4). They are the largest white blood cells with variable shape. The nuclei were extremely variable but usually have lacy chromatin (Figure 1e). The cytoplasm was blue-gray and contained variable size of vacuoles (Figure 1e). Monocytes stained moderately positive with SBB with faintly black, small granules scattered in the cytoplasm (Figure 3d). They were moderately to strongly positive with ANAE showing red-brown fine granular pattern (Figure 3l), but they were negative for PAS (Figure 3h) and b-glu. Under SEM, monocyte surfaces revealed more smooth membrane than those of neutrophil (Figure 7b) with deep fissure and micropores. Ultrastructurally, monocytes showed variable shape of nuclei with several mitochodria and pseudopodia (Figure 11c, 11d).

DISCUSSION

In this report, we described the light microscope, cytochemical features and ultrastructure of blood cells in Asiatic black bear. Although the erythrocytes in bears has special features such as uniform in shape with central pallor similar to those of dog, they are larger in mean diameter (Jain, 1993) and easy to form rouleaux. The RBC parameters of Asiatic black bear in this study was similar to but had lower number of leukocytes than those of Asiatic black bear in the zoo of Czechoslovakia (Pospisil *et al*., 1987). There was no blood parasite found in Asiatic black bear of this study while there was a report of filarid worm (*Dirofilaria ursi*) found in the esophageal and tracheal connective tissue of the male Asiatic black bear on Kyushu island (Yokohata *et al*., 1990).

Ultrastructure of platelets in Asiatic black bear were similar to those in bovine (Fern, 2000) and African elephant (Du Plessis and Stevens, 2002). Platelets in Asiatic black bear were negative for SBB which were the same as those in bovine, cat, dog, horse and green sea turtle (Raskin and Valenciano, 2000). Platelets in Asiatic black bear positively stained with ANAE which were similar to those of dog that were also positively stained with non-specific esterase (Raskin and Valenciano, 2000). And they were similar to platelets in Asian wild dog that were positive with ANAE and b-glu (Salakij *et al.*, 2000). So the ANAE stain would be

useful to differentiate megakaryocytic leukemia in the bears when using with the other non-specific esterase stains like those in human (Apibal, 1987).

The neutrophils in Asiatic black bear contained large granules that were unstained similar to vacuoles under light microscopy that were characteristic of neutrophils in Asiatic black bear. These vacuoles were large and variable in electron density as shown by TEM. This characteristic was not found in goat (Kramer, 2000) or in reindeer (Henkel *et al.*, 1999).

Characteristic features of basophils in Asiatic black bear were similar to those in dog; including having a long polymorphonuclear-shaped nucleus that is longer than those of neutrophil and thinner than those of most monocyte (Willard *et al.*, 1994). Basophils of Asiatic black bear are the same size as neutrophils but smaller than eosinophils (Table 2). These findings may be useful in differentiation of degranulated basophils from netrophils in Asiatic black bear.

The strongest reactivity of ANAE was found in eosinophils which was different from eosinophils in Asian wild dogs that were negative for ANAE (Salakij *et al.*, 2000). The bar-shape structures found in some granules of the eosinophil in Asiatic black bear by TEM were different from eosinophils of dog, cat (Young, 2000), goat (Kramer, 2000) and reindeer (Henkel *et al.*, 1999).

In the Asiatic black bear, granulocytes and monocytes stained with SBB. These findings are useful in differentiating acute myelogenous leukemia from acute lymphoblastic leukemia like those in human (Apibal, 1987). ANAE, AcP and b-glu staining characteristics of lymphocytes were similar to those reported in human that can differentiate T-lymphocytes (dot staining) from non T-lymphocytes (negative or fine granular staining) (Apibal, 1987; Khemtonglang *et al.*, 1997).

The basophils in Asiatic black bear were strongly positive with SBB and ANAE which is similar to reindeer basophils (Henkel *et al.*, 1999). The basophils in Asian wild dog were stained strongly positive with SBB, ANAE and b-glu (Salakij *et al.*, 2000). So in the Asiatic black bear, the SBB and ANAE are useful in the diagnosis between basophilic leukemia and megakaryocytic leukemia.

The ultrastructure of basophils in Asiatic black bear showed smaller granules than those in goat (Kramer, 2000) and in reindeer (Henkel *et al.*, 1999). The ultrastructure of lymphocytes and monocytes in Asiatic black bear were not different from those in pig (Steffens III, 2000) and in reindeer (Henkel *et al.*, 1999). Under SEM, the eosinophils revealed large granule contour which were easy to differentiate from basophils and neutrophils. This is the first report on the surfaces of blood cells in Asiatic black bear that could demonstrate abnormal shape of red blood cells and differentiation of white blood cells.

CONCLUSIONS

The neutrophils in Asiatic black bear contained large granules that were unstained and similar to vacuoles under light microscopy. This is characteristic of neutrophils in Asiatic black bear. The basophils in Asiatic black bear contained many small granules that not stained metachromatic with modified Wright stain so their nuclear outline were clearly defied. The results of this study provide more information on the morphology, cytochemical staining and ultrastructural characteristics of blood cells in Asiatic black bear. This information adds to our understanding of blood cells in healthy Asiatic black bears.

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