

Enzyme histochemistry and AgNOR numbers in the peripheral blood leukocytes of 6 month-old Kangal bred Anatolian shepherd dogs

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SUMMARY

The determination of peripheral blood lymphocyte (PBL) counts and the enzyme profile of PBL as reference values would be the first step to identify markers of lymphocyte differentiation and / or lymphoproliferation. In healthy, 6 month-old Kangal Shepherd dogs, PBL percentages, enzyme activities (α -naphthyl acetate esterase (ANAE), acid phosphatase (ACP-ase), β -glucuronidase (BG-ase) and N acetyl β -glucosaminidase (NABG-ase)) and some characteristics of silver stained nucleolus organizing regions (AgNOR parameters) were evaluated by histochemical methods. PBL percentage in females was significantly lower when compared to males ($p < 0.05$). Mean ANAE-positivity of PBL in males was 63.13 % whereas the positivity was slightly lower in females (60.75 %). The percentages of ACP-ase, BG-ase and NABG-ase positive lymphocytes of both genders were close similar. Values were respectively 39.37 %, 55.11 % and 52.45 % in males, and 39.12 %, 52.73 % and 51.37 % in females. There was no significant difference ($p > 0.05$) in mean AgNOR number nor in AgNOR diameters in PBL between males and females. Nucleus diameters and AgNOR diameter / nucleus diameter ratios of both genders were also quite similar. It was concluded that tabulated data as reference sources should be established for enzymatic positivity profiles and for AgNOR parameters in different age groups of Kangal breed and in other popular dog breeds. These data might be useful in early diagnosis of lymphoproliferative disturbances and in viral diseases caused by lymphotroph viruses and in the planning of therapeutic protocols, by using relatively cheaper techniques.

KEY-WORDS : lymphocyte - enzymes - AgNOR -histochemistry - dog.

RÉSUMÉ

Détection histochimique des enzymes et des AgNOR dans les leucocytes sanguins de bergers d'Anatolie (type Kangal) âgés de 6 mois. Par E. SUR, I. CELIK, Y. OZNURLU, M. FARUK AYDIN, I. SEN et H. OZPARLAK.

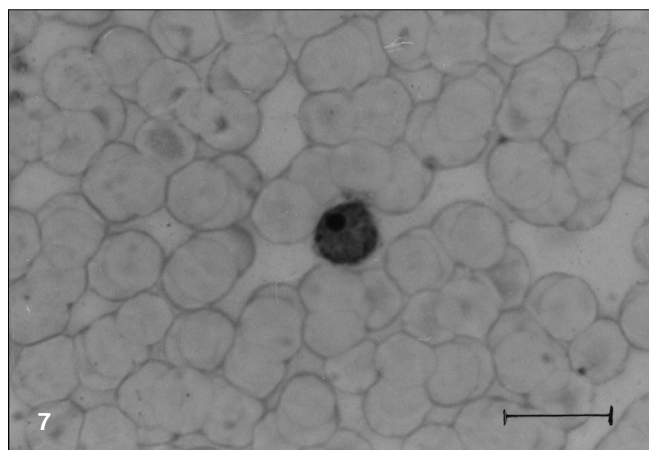
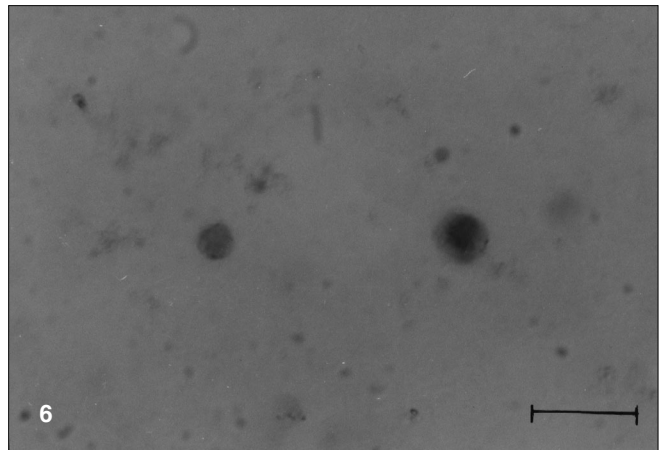
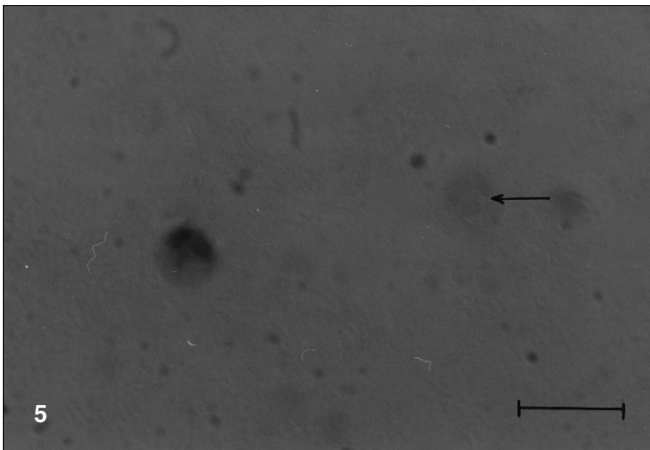
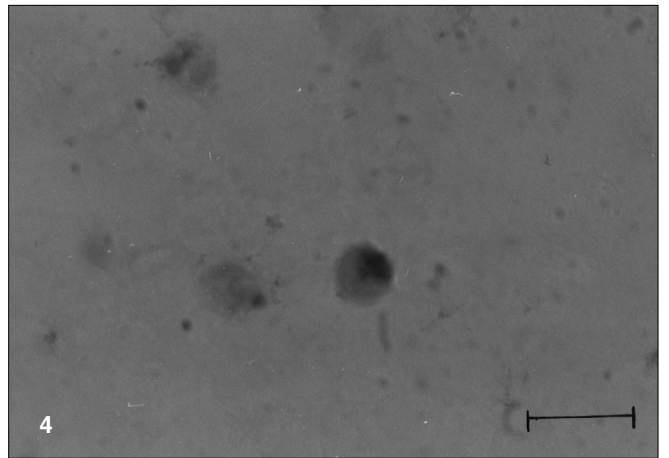
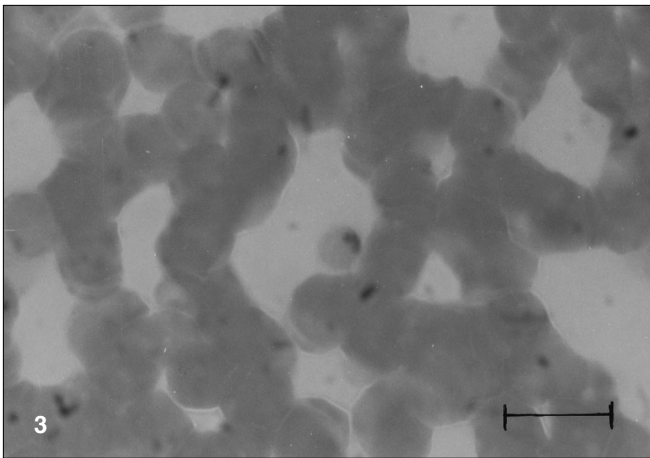
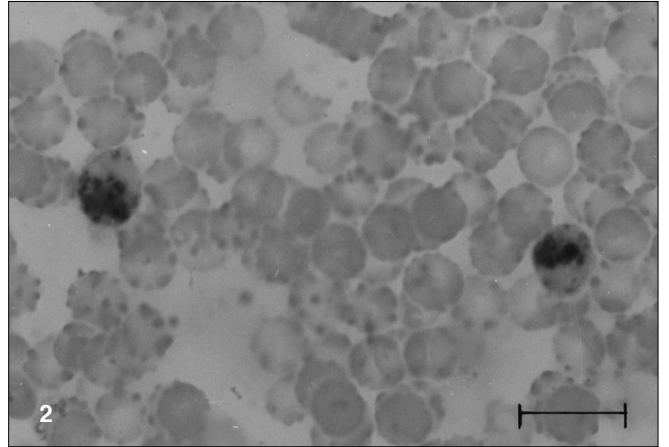
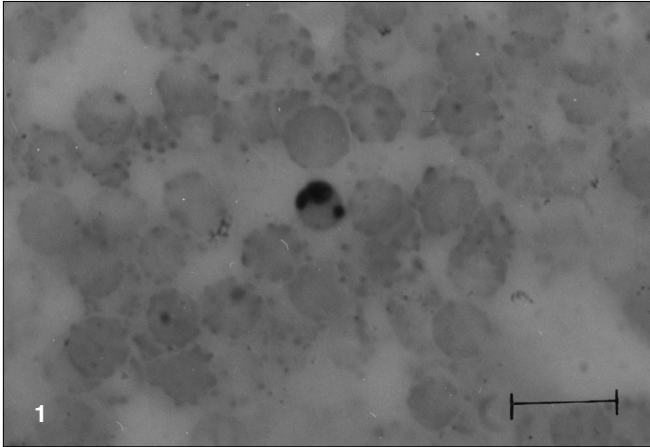
L'établissement de valeurs de référence du nombre de PBL et de leur profil enzymatique est la première étape nécessaire à l'identification de marqueurs de différenciation lymphocytaire et / ou de lymphoprolifération. Le pourcentage de PBL, plusieurs activités enzymatiques (α -naphthyl acetate esterase (ANAE), phosphatase acide (ACP-ase), β -glucuronidase (BG-ase) et N acetyl β -glucosaminidase (NABG-ase)) et des caractères nucléolaires (AgNOR) ont été mesurés par des techniques histochimiques chez des chiens de berger du Kangal, en bonne santé, âgés de 6 mois. Le pourcentage de PBL chez les femelles était significativement plus faible que chez les mâles ($p < 0.05$). Chez les mâles, le taux moyen de positivité en ANAE était de 63.13 % tandis qu'il était légèrement plus faible chez les femelles (60.75 %). Les pourcentages de lymphocytes positifs en ACPase, en BG-ase et en NABG-ase étaient tout à fait équivalents dans les 2 sexes. Les valeurs mesurées étaient respectivement de 39.37 %, 55.11 % et de 52.45 % chez les mâles et de 39.12 %, 52.73 % et 51.37 % chez les femelles. Aucune différence significative entre mâle et femelle n'a été obtenue en ce qui concerne le nombre ou le diamètre des AgNOR des PBL ($p > 0.05$). Les diamètres nucléaires et les rapports diamètre de l'AgNOR / diamètre nucléaire étaient identiques quelque soit le sexe. En conclusion, des valeurs de référence des profils enzymatiques et des caractères des AgNOR devraient être établies pour différentes classes d'âge des bergers du Kangal et pour d'autres races de chien tout aussi fréquentes. Ces données pourraient permettre d'établir un diagnostic précoce des cas de lymphoprolifération et des maladies virales dues à des virus lymphotropes et dans la mise en place de protocoles thérapeutiques, par des techniques relativement peu onéreuses.

MOTS-CLÉS : lymphocyte - enzymes - AgNOR - histochimie - chien.

Introduction

Enzyme cytochemical and histochemical tests have practical value for the study of immature, blast cells and lymphocytes since Romanowsky-stained films might be insufficient

to identify differentiation and maturation features of the cells [2, 7]. Peripheral blood lymphocyte (PBL) counts and their enzymatic profile may change in the human [7] and bovine leukemia [23], Marek's disease in the chicken [16, 21] and lymphotropic viral diseases, such as canine distemper [1, 46] and caprine encephalo-arthritis [22].



Results of BASSO *et al* [3] have revealed that acid phosphatase (ACP-ase) is first gained by early thymocytes, and following, the enzymes α -naphthyl acetate esterase (ANAE), beta-glucuronidase (BG-ase), N-acetyl- β -glucosaminidase (NABG-ase) and β -naphthyl butyrate esterase (NBE) are acquired at the later phases of T-lymphocyte maturation in humans. Their results have also showed that most of the mature, immunocompetent, peripheral blood T-lymphocytes show strong enzymatic activity for both ANAE (78 %) and ACP-ase (90.8 %). However, ACP-ase positivity is also found at high rates (96.0 %) in immunologically immature thymocytes and lymphocytes at the fetal period. ANAE positivity has largely been used as a T-cell marker since KAJIKAWA *et al* [23] in the cattle, MUELLER *et al* [38] in the mouse, MAITI *et al* [33] in the chicken, WULFF *et al* [58] in the dog have showed that ANAE positivity reflects the T-lymphocyte frequency of the PBL and localization pattern in the canine lymphoid tissues [4, 58]. WULFF *et al* [58] referred only to the focal, dot-like ANAE positivity as a T-cell marker and found 52-78 % positivity in adult, 0.8-7 years old dogs. Nevertheless, NAKASE and KOBAYASHI [39] have reported that 56.9 % of canine blood lymphocytes were positive for ANAE regardless ages of the animals.

ACP-ase is a member of acid hydrolases. The enzymatic activity is gained at early stages of T-lymphocyte maturation in the human thymus [3]. The reaction is positive in fetal thymocytes and it also persists in some of the mature T-lymphocytes localised in the thymus-dependent, T-cell areas of lymphoid tissues from humans and rodents [6, 7, 24]. The enzyme is of diagnostic value in the differential diagnosis of lymphoproliferative disorders [57] and its reaction dramatically increases in almost all acute and chronic T-cell lymphoproliferations in humans [7].

Nucleolus organizing regions (NORS) are the loops of DNA containing ribosomal RNA genes [5, 9, 45] and proteins in these regions are easily visualized as silver-stained black dots (AgNORS) in the cell nucleus with colloidal silver since they are argyrophilic [9]. The size of the silver deposit on the NOR is thought to be positively correlated to the degree of transcriptional activity of the cell [55, 56, 60] and suggested to reflect nucleolar activity [36, 43]. A positive correlation has been reported between increased size and / or numbers of AgNORS and cellular proliferative activity of tumoral cells [9, 15]. Number and size of AgNORS per nucleus and mean AgNOR number of a given patient has been suggested to be a reflection of some certain tumors and

their malignancy [5, 17, 34, 41, 42, 47, 53], and it has also been suggested that AgNORS-related parameters are one of the most powerful variable predicting survival in patients with pharyngeal carcinoma, multiple myeloma, male breast and prostate carcinomas [42, 50]. However, nerve cells, glandular cells, stimulated monocytes and T-lymphocytes have a very high demand for ribosomes but divide slowly or not at all [43] and display higher nucleolar activity [55].

In the present study, percentages of lymphocytes, the positivity rates of ACP-ase, BG-ase, NABG-ase and ANAE in PBL of the clinically normal, 6 month-old Kangal Shepherd dogs were determined. Some of the AgNOR parameters of PBL were also determined.

Materials and Methods

A) ANIMALS

Blood samples collected from 16 of healthy Kangal bred of Turkish Shepherd dogs aged 6 months-old. The dogs were kept under feeding and management conditions of Research Farm of Selçuk University, Veterinary Faculty. The peripheral blood samples were taken in heparinized (10 IU ml⁻¹ ml blood, Liquemine flc., Roche) tubes from cephalic antebrachial vein. From each animal, two smears for each staining procedure were prepared and air dried and were stained with May Grunwald-Giemsa according to standard staining procedures [28].

B) DEMONSTRATION OF ANAE

ANAE demonstration was performed according to WULFF *et al* [58] with minor modifications. Briefly, blood smears were fixed in cold glutaraldehyde-acetone (pH 4.8) for three minutes at -10°C. After fixation, the smears were rinsed three times in distilled water. Incubation solution was prepared by mixing 20 mg of substrate, alpha naphthyl-acetate (Sigma) dissolved in 0.8 ml acetone (Merck), 4.8 ml of hexazotized pararosaniline [(hexazotization was performed by mixing equal volumes (2.4 ml each) of 4 % sodium-nitrite (Merck) and 2 % pararosaniline (Merck)] and 80 ml of buffered phosphate saline (pH 5). Final pH of the incubation solution was adjusted to 5.8 with 1N NaOH, and then the solution was filtered. After one-hour-incubation at 37°C, the smears were rinsed 3 times in distilled water, and then nuclei stained for 20 minutes in 1 % methyl-green prepared in acetate buf-

LEGEND OF OPPOSITE PHOTOGRAPHIES :

- FIG.1. — A localized granular ANAE positive lymphocyte with reddish-brown cytoplasmic granules. ANAE demonstration, Bar :10 μ m.
- FIG. 2. — Diffuse granular ANAE positive monocytes with reddish-brown cytoplasmic granules localized in the nuclear indentation area. ANAE demonstration, Bar : 10 μ m.
- FIG.3. — An ACP-ase lymphocyte with pinkish-red cytoplasmic granules. ACP-ase demonstration, Bar :10 μ m.
- FIG. 4. — An ACP-ase positive monocyte with diffuse pinkish-red stained granules localized in the nuclear indentation area. Neutrophils are weak positive. ACP-ase demonstration, Bar :10 μ m.
- FIG.5. — A BG-ase positive monocyte with diffuse pinkish-red staining localized in the nuclear indentation area. Neutrophils are negative for the enzyme. ACP-ase demonstration, Bar :10 μ m.
- FIG. 6. — NABG-ase positivity in monocytes. A positive monocyte with diffuse pinkish-red staining localized in the nuclear indentation area. A lymphocyte with weak positivity with a small granular reaction product is also seen. NABG-ase demonstration, Bar :10 μ m.
- FIG.7. — AgNOR in the nucleus of a small lymphocyte. AgNOR staining, Bar :10 μ m.

fer (pH 4.2). Control specimens were prepared by incubating the smears in incubation solution without alpha naphthyl-acetate.

C) DEMONSTRATION OF ACP-ASE

ACP-ase was demonstrated with the method of GOLDBERG and BARKA [12] with minor modifications. In the technique, blood smears were fixed in formal-calcium at +4°C for 10 minutes and following, the smears were rinsed in distilled water three times. Incubation solution was prepared by mixing 10 mg of naphthol AS-BI phosphate (Sigma) dissolved in 1 ml of N, N-dimethyl formamide (Sigma), 13 ml distilled water and 1,6 ml hexazotized pararosaniline (prepared as in the ANAE incubation solution) and 5 ml Michaelin's veronal acetate buffer (pH 5). Final pH of the solution was adjusted to 5.0 with 1 N NaOH, and the solution was filtered. After for one-hour incubation at 37°C, the slides were rinsed 3 times in distilled water, and nuclei stained for 20 minutes with 1 % methyl-green prepared in acetate buffer (pH 4.2). Incubation solution of control smears did not contain naphthol AS-BI phosphate.

D) DEMONSTRATION OF BG-ASE

BG-ase was demonstrated according to LORBACHER *et al* [31]. Briefly, air dried blood smears were fixed in formal-calcium at +4°C for 3 minutes. Following, the smears were incubated in the incubation solution for 40 minutes at 37°C. The solution was prepared by mixing 5 ml substrate solution (210 mg of naphthol AS-BI glucuronide (Sigma) in 50 ml of distilled water), 0.3 ml of the hexazotized pararosaniline and 5 ml distilled water. At the end of incubation, the smears were rinsed twice in distilled water and counter stained with 1 % methyl green prepared in acetate buffer (pH 5.2).

Control specimens were prepared by incubating the smears in incubation solution without naphthol AS-BI glucuronide.

E) DEMONSTRATION OF NABG-ASE

NABG-ase activity was obtained in the smears fixed in formaldehyde vapor for 3 minutes. The smears were rinsed twice in distilled water and incubated for 30 minutes at 37°C in incubation medium prepared according to BASSO *et al* [3]. Three mg of naphthol AS-BI N-acetyl- β -glucosaminide (Sigma) was dissolved in 0.5 ml ethylene glycol monomethyl ether (Merck) and 5 ml 0.1M citrate buffer (pH 5.2) added. The incubation solution was prepared by mixing 0.6 ml of the hexazotized pararosaniline with 5.5 ml of the substrate solution. Final pH was adjusted to 5.2 with 0.1N NaOH.

Incubation solution of control smears did not contain naphthol AS-BI N-acetyl- β -glucosaminide.

F) STAINING THE AgNORS

Air dried smears were fixed in Carnoy's fluid and staining was done using a solution consisting of one volume of 2 % gelatin in 1 % aqueous formic acid and two volumes of 50 % silver nitrate. The staining was performed at 37°C in the dark for 10-12 minutes by frequently controlling light microscope [29, 40].

G) EVALUATION OF THE STAINED SPECIMENS

All specimens were examined under light microscope (Leitz, Laborlux 2). May Grünwald-Giemsa stained smears were used to determine PBL ratio of the animals. ACP-ase, BG-ase and NABG-ase positivities were determined as pinkish reaction products. On each specimen the lymphocytes having 1-3 pinkish cytoplasmic granules were concerned as positive for enzyme investigated. Enzymatic positivity for ANAE was characterized as localized and granular reddish-brown reaction product in the positive lymphocytes (Fig. 1), whereas the positivity pattern of monocytes was diffuse and non granular (Fig. 2). In each of the ACP-ase, ANAE, BG-ase and NABG-ase demonstrated specimens, 200 lymphocytes were counted and positivity rates were expressed as percent of the counted cells. In AgNOR stained specimens, AgNORs in 100 cell nuclei were counted and the results were expressed as AgNOR number per nucleus. Two diagonal measurements were also performed with a linear ocular micrometer on the cell nuclei and their AgNORs, and both mean nucleus diameter and mean AgNOR diameter were calculated for a given cell. AgNOR diameter/cell nucleus diameter ratio was also calculated, by using the obtained data.

H) STATISTICAL ANALYSES

ACP-ase and ANAE positivities following transforming, whereas AgNOR parameters directly were analysed with one-way ANOVA (Minitab for Windows, Release 9.2). Results were considered as significant when p values were less than 0.05.

Results

A) PERIPHERAL BLOOD LYMPHOCYTE PERCENTAGES

Mean PBL percentage of the females was significantly ($p < 0.05$) lower when compared to those of the males (Table I).

B) ANAE POSITIVITY IN LEUKOCYTES

ANAE-positivity was observed in lymphocytes and monocytes. ANAE-positive lymphocytes displayed a dot like positivity pattern characterized with 1-3 reddish-brown cytoplasmic granules (Fig. 1). Monocytes gave a diffuse, fine granular reaction (Fig. 2). Control specimens did not displayed enzymatic reaction. Mean ANAE-positivity of PBL in males was 63.13 %, whereas the positivity was slightly lower, 60.75 % in females. However, the difference between mean values of the genders was not statistically significant ($p > 0.05$, Table I).

C) ACP-ASE POSITIVITY IN LEUKOCYTES

A large number of lymphocytes and monocytes have showed strong ACP-ase positivity (Fig. 3 and 4). The lymphocytes having 1-3 pinkish cytoplasmic granules were **conse-**

red as ACPase-positive (Fig. 3). Neutrophils displayed a weak positive reaction for ACP-ase (Fig. 4). Control specimens did not displayed enzymatic reaction. However, the enzymatic reaction was commonly present in lymphocytes. In the control specimens there was no specific enzymatic reaction in any cell type. Results of enzyme histochemical evaluations are present in Table I. Mean percentages of ACP-ase positive lymphocytes of both of the genders was close similar. The values were 39.37 % for males and 39.12 % for females (Table I).

D) BG-ASE POSITIVITY IN LEUKOCYTES

Vast majority of lymphocytes and monocytes displayed a weak, non granular staining whereas, neutrophils did not show any detectable BG-ase activity. Positivity pattern of monocytes was diffuse granular (Fig. 5). Lymphocytes showed similar positivity patterns and rates to ANAE (Table I). Control specimens did not displayed enzymatic reaction. The difference between the positivities of the genders was not significant ($p > 0.05$).

E) NABG-ASE POSITIVITY IN LEUKOCYTES

NABG-ase was peculiar to monocytes and lymphocytes (Fig. 6). Reaction product in lymphocytes was quite similar to those of the BG-ase. NABG-ase positivity percentage was lower than those of the ANAE and BG-ase. However, the rate was higher than ACP-ase. Both males and females displayed

similar positivity rates (Table I). In control specimens, enzymatic reaction products were not observed.

F) RESULTS OF AgNOR STAINING PARAMETERS

AgNORs were observed as black patches having irregular shape in the cell nuclei (Fig. 7). There were no significant ($p > 0.05$) differences in mean AgNOR numbers and AgNOR diameters of PBL between the males and females. Nucleus diameters and nucleus diameter / AgNOR diameter ratios of both genders were quite similar (Table II).

Discussion

Enzyme histochemical techniques have largely been used to identify some certain haematological disorders, specifically in the diagnosis and classification of both human [7] and bovine leukemias [23].

Non specific esterases are widely distributed in various types of cells. Cytochemical esterase activity is commonly used to differentiate types of leukocytes and leukemia cells [18]. The ANAE cytochemical reaction is often applied to the study of leukaemic cell populations. In normal blood samples it helps to distinguish between T-lymphocytes and monocytes because of different pattern of the reaction and the different sensitivity to natrium floride (NaF). Furthermore, the reaction in PBL is resistant to NaF, while that of the monocytes is NaF-sensitive [14, 31].

Gender of the animals	Mean lymphocyte ratio of the peripheral blood (%) (X±SEM)	Mean ANAE-positivity (%) (X±SEM)	Mean ACP-ase positivity (%) (X±SEM)	Mean BG-ase positivity (%) (X±SEM)	Mean NABG-ase positivity (%) (X±SEM)
Males (n=8)	38.125±0.910	63.13±1.87	39.375±0.905	55.11±1.57	52.45±0.87
Females (n=8)	35.750±0.491	60.75±1.29	39.125±0.915	52.73±1.32	51.37±1.23
Significance	*	N.S.	N.S.	N.S.	N.S.

* $p < 0.05$

TABLE I. — Enzymatic positivity rates of peripheral blood lymphocytes of the animals.

Gender of the animals	Mean AgNOR number per nucleus(X±SEM)	Mean nucleus diameter (µm) (X±SEM)	Mean AgNOR diameter (µm) (X±SEM)	Mean AgNOR diameter / nucleus diameter ratio (%) (X±SEM)
Males (n=8)	2.301±0.048	5.325±0.065	0.8587±0.020	0.15625±0.0026
Females (n=8)	2.271±0.045	5.275±0.065	0.8450±0.018	0.15625±0.0032
Significance	N.S.	N.S.	N.S.	N.S.

TABLE II. — AgNOR parameters of the animals.

In the normal peripheral blood, there is a good correlation between the proportion of T-cells and percentage of ANAE-positive lymphocytes in humans [8, 14, 38], in the chicken [33], in the cattle [23, 59], in the mouse [38] and in the dog [58]. In this study, 63.13 % of PBL in males and 60.75 % in females displayed positive reaction for ANAE. Previous researchers [20, 46, 58] have reported different enzymatic positivity rates for canine PBL. WULFF *et al* [58] have reported that 56-58 % of the peripheral blood lymphocytes were positive for ANAE in healthy dogs. SEN *et al* [46] have studied on 8 healthy mix-bred dogs, aged 6-8 weeks and reported 46.74 % ACP-ase, 62.64 % ANAE, 49.43 % NABG and 14.77 % BG positivities. The differences might possibly be arisen from the differences of ages, species, genders of the dogs and environmental conditions.

In the lymphoid leukosis of the cattle [23], in Marek's disease of the chicken [16, 21], in distemper disease of the dog [1, 46] significant changes have been reported either in PBL percentages or their enzymatic positivity rates. Besides, IWATSUKI *et al* [19] have showed a definite lymphoid cell depletion from the lymphoid organs at the early stage of canine distemper virus (CDV) infection. In another study, SEN *et al* [46] have assayed the changes in enzymatic-positivity profile of PBL through the development of experimentally induced CDV infection. They have observed a sharp decrease in the percentages of ACP-ase, ANAE, NABG and BG. The decrease started on the day 2, gradually continued and reached at their lowest levels at 9th day. The most prominent declines were in the ACP-ase and ANAE positive lymphocytes. The researchers [46] have claimed that ACP-ase and ANAE enzyme positivities might be used as a laboratory aid in the early diagnosis of CDV infection.

IZCI *et al* [20] have monitored the effects of ophthalmically administrated 2 % cyclosporine on the total percentage and ANAE positivity rates of PBL of the dogs with keratoconjunctivitis sicca (KCS). Baseline levels of the dogs were 56.17 % for total lymphocytes and 71.67 % for ANAE positive lymphocytes. The researchers [20] have revealed that the total percentage and ANAE positivity of PBL gradually decreased (22.67 % and 36 % respectively) in 2 % cyclosporine treated dogs at 60th day of the experiment.

ACP-ase positive granules were also found in neutrophils, eosinophils, and monocytes [30]. Lymphocytes and monocytes have generally more ACP-ase activity than neutrophils. In human and rodent lymphoid tissues, ACP-ase activity has been demonstrated in lymphocytes which localized in T-lymphocyte areas [52]. However, it is considered that ACP-ase is related to B-lymphocyte maturation and regarded as a B-cell marker in the chicken [10, 11, 49, 51] since the enzyme is commonly present in lymphocytes originated from bursa of Fabricius. In this study, ACP-ase positivity rates of PBL in male and female dogs were quite similar (39.37 % for males and 39.12 % for females) although the rates were lower than those of SEN *et al* [46]. However, they have reported 46.74 % positivity rate for ACP-ase in 8 healthy mix-bred dogs, aged 6-8 weeks. The difference might have arisen from the differences of the ages, breeding and environment of the animals used in both experiments.

ACP-ase is of diagnostic value in the differential diagnosis of lymphoproliferative disorders [57] and its reaction dramatically increases in almost all acute and chronic T-cell lymphoproliferations in humans [7]. Strong ACP-ase activity in B-cell prolymphocytic leukaemias is also observed and one-third of the cells display a positive ACP-ase reaction [6]. ACP-ase activity also increases after lymphocyte transformation [7, 12] with phytohemagglutinin (PHA) and during the transition from monocyte to tissue macrophage [24, 30].

LORBACHER *et al* [31], have reported that vast majority of human neutrophils and eosinophils showed non granular BG-ase staining. On the contrary, MACHIN *et al* [32] have found BG-ase activity in neutrophils, eosinophils, lymphocytes, monocytes and plasma cells of humans. In this study, all canine monocytes and a greater proportion (55.11 % in males and 52.73 % in females) of PBL have showed BG-ase positivity. The results are relatively higher than the results reported by SEN *et al* [46]. However, their material was 8 healthy mix-bred dogs, aged 6-8 weeks. The difference might be arisen from the conditions, age and bred of the animals used in both studies. Staining pattern was localized granular in lymphocytes and diffuse, non granular in monocytes those quite similar to that of the ANAE staining pattern. Both normal and neoplastic B cell populations were dominantly BG-ase negative, while both normal and neoplastic T cells were BG-ase positive. It has been suggested [32] that BG-ase positivity rate closely corresponds to E rosette formation and reflects T cell proportions in the PBL of humans. Nevertheless, the results of this study have revealed that BG-ase positivity was relatively lower than ANAE in the dogs from both genders. The latter has been regarded as an enzymatic T cell marker in the dogs [58]. Besides, MACHIN *et al* [32] have pointed out that there were several limitations such as, BG-ase positivity of some activated B cells, variable expression of the activity by neoplastic cells of chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and the T cell derived malignancies, and less practicability of demonstration method than ANAE that gives more precise results [58]. Significantly decreased BG-ase activity in CLL has been reported, whereas the activity increased in Hodgkin's disease [31]. Besides, KLOBUSICKA *et al* [25] have showed a significant increase in both BG-ase positivity and AgNOR quantity in the proliferation activity of leukaemic cells and the authors [25] have suggested that these parameters might be of value in monitoring the risk groups of leukaemia patients.

Mean NABG-ase positivities were 52.45 % in males and 51.37 % in females in this study. NABG-ase is a lysosomal enzyme, since the reaction granules of NABG-ase have a general cytological localization quite similar to that of other lysosomal enzymes in kidney and liver. However, the enzyme has less importance in the leukocyte enzyme histochemistry.

Significant differences between the genders in AgNOR number per nucleus, AgNOR diameter and AgNOR diameter/nucleus diameter ratio were not observed in this study. AgNOR numbers of PBL was found as 2.30 and 2.27 in males and females respectively. AgNOR diameters were

0.85 µm in males and 0.84 µm in females. AgNOR diameter/nucleus diameter ratio in both males and females was 0.15 %. HUNG *et al* [17] have suggested that measurement of cell kinetics, employing AgNOR and proliferating cell nuclear antigen (PCNA) detection as proliferation markers, might provide a valuable means for differentiating benign canine tumors from malignant tumors. Besides, AgNOR count has been assumed as a reflection of transcriptional activity of interphase or mitotic cells and degrees of malignancy of tumors [5]. Additionally, RUSSEL *et al* [44] have reported that occurrence of AgNORs were related to proliferative activity of the cell but an increase in AgNOR cluster size rather than elevated AgNOR cluster numbers was the major feature [5, 13, 35, 46, 52]. Accordingly, METZE *et al* [34] have concluded that the AgNOR pattern in CLL describes the cell kinetic changes during the evolution of the disease and is a prognostic factor for tumor reduction after treatment. Besides, results of previous studies [2, 26, 27, 32, 33, 39, 48, 52, 54] have showed a close relationship between immaturity and proliferative ability of blast cells in both AML and T-ALL patients and the increase in AgNOR numbers. It is not known whether there is any relation of enzymatic positivity rates to AgNOR parameters of PBL. However, KLOBUSICKA *et al* [25] have found a close relationship between increased AgNOR quantity and BG-ase activity in the proliferative activity of leukemic cells in T-ALL, and thus the parameters are of value in monitoring the risk groups of leukemic patients.

GROTTO *et al* [13] have reported that mean numbers of AgNOR clusters and dots per cell nucleus were 2,87 and 0,23 respectively, in bone-marrow cell of acute myeloid leukemia (AML) patients. The data were lower than normal values, 3,27 and 0,34 for mean number of AgNOR cluster and dots respectively. MOURAD *et al* [37] have pointed that 5 or more AgNOR dots in per bone-marrow cell were a reflection of proliferative activity in B-cell chronic lymphocytic leukemia (B-CLL) patients. AgNOR pattern has a clinical importance in some leukemic diseases. In a study on bone-marrow aspirate by SHOME and KHURANA [48] small (< 3 µm) and dots AgNOR pattern in lymphoblasts was specific for acute non-lymphoblastic leukemia (ANLL), whereas larger and blebs or combination of the two types of myeloblasts were specific for ALL. In dogs, AgNOR staining has been reported to be a useful method in the discrimination of grade II or III mast cell tumors from grade I, perianal gland carcinomas from adenomas (or hyperplasia), fibrosarcomas from non-fibrosarcoma tissues. The mean AgNOR cut-off points were 6.0, 14.1, 9.4, and 8.8 AgNOR number Per nucleus respectively [17].

In the present study, PBL ratio and ANAE, ACP-ase, BG-ase, NABG-ase positivities of PBL and some of the AgNOR parameters of PBL were determined in 6 months-old, Kangal bred Turkish Shepherd dogs. It is a well known phenomena that aging results in decreased immune functions as a result of quantitative and functional declines in immune reaction parameters. The authors strongly stress that tabulated data of young and old dogs covering all age groups as a reference source should be established for both each enzymatic reactions and AgNOR parameters. This may facilitate early dia-

gnosis and planning of treatment procedures of lymphoproliferative disturbances and lymphotropic viral diseases, by using relatively cheaper techniques. Also, these techniques has the advantage of pre-selecting the complicated cases which need further, expensive, complicated diagnostic procedures.

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