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Determination of alpha-naphthyl acetate esterase (ANAE) activity in peripheral blood leukocytes of pregnant, adult, and kitten Angora cats

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Abstract: This study aimed to determine the alpha-naphthyl acetate esterase (ANAE) activity of peripheral blood leukocytes in pregnant, kitten, and adult Angora cats. In each group 9 healthy pregnant, adult, and kitten Angora cats constituted the material of the study. Optimum reaction was achieved after a 3 h incubation period at pH 5.8, by ANAE staining. Two types of reaction were observed in ANAE (+) T lymphocytes. The first reaction was a dot-like positivity pattern characterized by the presence of large granules, while the other was a granular positivity pattern characterized by the presence of 3 to 5 small granules. B lymphocytes reacted negatively in ANAE staining. The rate of ANAE (+) lymphocytes in pregnant, kitten, and adult Angora cats was 62.89 ± 1.29%, 68.37 ± 1.22%, and 77.71 ± 1.63%, respectively. In conclusion, the lowest rate of ANAE-positive lymphocytes was detected in the pregnant cats, depending on the maternal immune tolerance supported by hormonal mechanisms. An increased rate of ANAE-positive lymphocytes was detected in the kittens, and the highest rate was measured in the adults, in parallel with the development of the immune system.

Key words: ANAE activity, Angora cats, lymphocyte, pregnancy

1. Introduction

Esterase enzymes, belonging to the hydrolase enzymes, break down the ester bonds and, thereby, catalyze the hydrolysis of esters. Esterases are classified under 2 groups, nonspecific esterases and specific esterases. Esterases, which hydrolyze simple esters such as naphthylacetate, are classified as nonspecific esterases. Nonspecific esterases are widely distributed in the body and are found in several different types of cells. Practical staining methods, based on cytochemical esterase activity, are commonly used to demonstrate leukocytes and leukemia cells, which cannot be differentiated by the conventional Romanowsky staining procedure (1). Alpha-naphthyl acetate esterase (ANAE), a nonspecific esterase, is also used as a distinguishing marker, as it produces a dot-like positivity pattern in sites where mature T lymphocytes are present at high levels (2). Several researchers have demonstrated its presence in T lymphocytes and absence in B lymphocytes (3–5). Environmental conditions, particularly pH and temperature, play an important role in ANAE activity. Depending on the acidity or alkalinity of the environment esterases may terminate their activity to a large extent, similar to other hydrolytic enzymes. Maximum and minimum pH values prevail for the optimum enzyme activity of all known esterases. Variations in the activation of esterases and pH alterations determine the characteristics of the enzyme activator complex (6). The optimum pH range for nonspecific esterase activity is between 5 and 8 (7).

Prothymocytes, which migrate from the bone marrow, enter the thymus via blood vessels found in the corticomedullary junction. After completing the 4 stages of maturation, these cells migrate from the subcapsular region to the cortex and then from the cortex to the medulla, eventually entering the blood circulation in the form of mature peripheral blood T lymphocytes (8). During this period the lymphocytes gain lysosomal enzymes such as ANAE. ANAE staining has demonstrated 2 types of positivity occurring in peripheral blood lymphocytes (9). The first, referred to as the dot-like positivity pattern, is characterized by the formation of one or several reddish-brown colored granules and is specific to mature T lymphocytes (10). The second type of positivity observed upon ANAE staining is the fine granular positivity pattern. Several researchers have reported that B lymphocytes react negatively for ANAE staining (11). Depending on

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the species and the staining method employed, ANAE positivity is observed in different forms in the other peripheral blood cells and platelets (Table 1).

Gestation is a physiological phenomenon characterized by the tolerance of the maternal immune system to paternal antigens expressed by the fetus. Under normal conditions, the semiallogenic embryo is obliged to develop strategies to protect itself from the attacks of the maternal immune system throughout gestation. Despite variations between animal species, all phenomena that restrict the reaction of the maternal immune system to the trophoblast cells, which carry paternal antigens and are formed in early gestation, are defined as maternal immune tolerance (19,20). The factor most significant for the development of maternal immune tolerance is the progesterone hormone, which has an immunosuppressive effect. The mode of action of progesterone, which is first secreted from the ovaries during the secretory phase of the sexual cycle, remains controversial. It is suggested that the progesterone receptors of T lymphocytes increase in number during the luteal phase of the sexual cycle and throughout gestation, and it is indicated that the progesterone hormone inhibits the proliferative activity of T lymphocytes (21).

The present study aimed to demonstrate alterations in ANAE activity of peripheral blood leukocytes of kitten, adult, and pregnant Angora cats, a local breed.

2. Materials and methods

Blood samples were collected from the vena cephalica of 27 healthy Angora cats bred at Kırıkkale University. The animal care and protocol used were reviewed and approved by the ethics committee of Kırıkkale University (28.04.2008/19).

Table 1. Distribution of ANAE positivity in the peripheral blood cells.

Researchers/animal species	M	E	N/H	P/T
Asti et al. (3) Rat[a], chicken, sheep, goat, cattle, horse[1], cat[1], dog[1a]	+	+	+	-
Yoruk et al. (4) Van cat	+	+	-	*
Asti et al. (12) Chicken	+	+	+	-
Sur et al. (13) Pheasant (*Phasianus colchicus*)	+	+	+	*
Ergun et al. (14) Ostrich (*Struthio camelus masaicus*)	+	-	-	-
Ergun et al. (15) Turkey	+	+	+	-
Sandıkçı et al. (5) Camel (*Camelus dromedarius*)	+	-	-	-
Özcan, (16) Angora rabbit	+	+	+	+
Donmez et al. (17) Kangal fish (*Garra rufa*)	+	+	+	-
Donmez and Sur (9) *Rock partridges* (*Alectoris graeca*)	+	*	+	-
Altunay et al. (18) Gazelle (*Gazella subgutturosa*)	+	+	-	-

*No information.

M: monocytes, E: eosinophil granulocytes, N/H: neutrophil/heterophil granulocytes, P/T: platelet/thrombocytes. 1: neutrophil granulocytes negative, a: thrombocytes positive.

Two blood smears were prepared from heparinized blood samples and were dried at room temperature. To determine the ANAE activity, blood smears were fixed in a glutaraldehyde–acetone solution at −10 °C for 3 min and then air-dried. Then the smears were immersed in an incubation solution of phosphate buffer and hexazotized pararosaniline (pH 5.0) containing the enzyme substrate 0.25% alpha-naphthyl acetate (11). Finally, the incubation solution was adjusted to pH 5.8. The blood smears were counterstained with 1% methyl green (pH 4.2) for 20 min (9).

The ratio of ANAE positivity in every smear was determined by counting 300 lymphocytes. The data was analyzed by one-way ANOVA (22). Duncan's multiple range tests were performed when F-values were significant. P-values less than 0.05 were considered significant.

3. Results

Two types of ANAE (+) reaction were observed in the T lymphocytes of the Angora cats following a 3 h incubation period at pH 5.8. The first type of positivity was the dot-like pattern, characterized by the presence of 1 to 2 large brown-red colored granules in the cytoplasm (Figure 1). The second type was a granular pattern identified by 3 to 5 smaller granules of the same color (Figure 1). No reaction was observed in the B lymphocytes (Figure 2). Certain monocytes presented with a diffuse granular positivity pattern. Neutrophil, eosinophil, basophil granulocytes, and platelets (Figure 3) negatively reacted with ANAE staining. The blood cell counts of the pregnant, adult, and kitten Angora cats are presented in Table 2.

4. Discussion

Esterases, which hydrolyze simple esters such as naphthyl acetate, are classified as nonspecific esterases. Nonspecific esterases are found in a wide range of cells, primarily mature T lymphocytes. ANAE, which is a lysosomal enzyme, is reported to give the cell a cytotoxic property (11). The main factors influencing enzyme activity are substrates, pH, and temperature. Thus, the pH levels at which enzymes are activated vary between animal species. To exemplify, the pH value at which ANAE is activated has been reported as 6.4 in Angora goats (3); 7.2 in chickens (2); 6.5 in dogs; 6.4 in pigs (23); 6.2 in horses and cattle; 6.4 in rats and cats (3); and 5.8 in camels (5), turkeys (15),

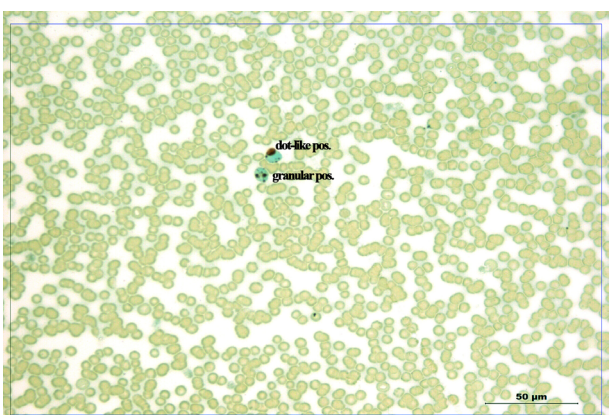

Figure 1. ANAE staining positivity types. Bar = 50 µm.

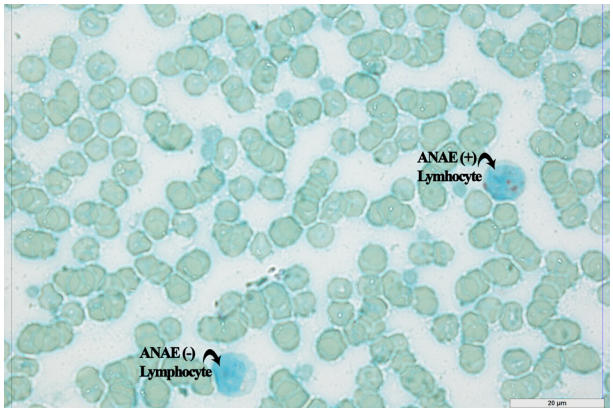

Figure 2. Reaction of peripheral blood lymphocytes with ANAE staining. Bar = 20 µm.

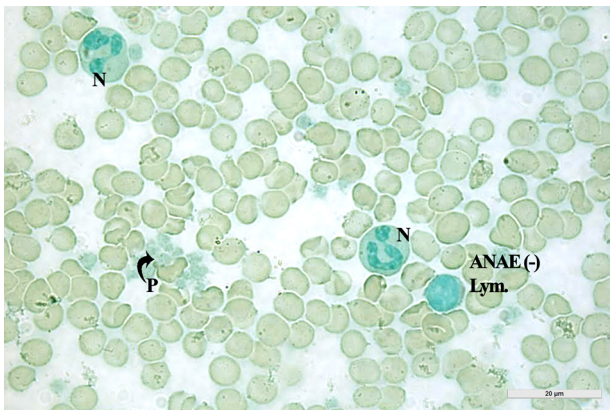

Figure 3. ANAE reaction in different blood cells. N: neutrofil, P: platelets. Bar = 20 µm.

Table 2. ANAE-positive peripheral blood lymphocytes rates in pregnant (n = 9), adult (n = 9), and kitten (n = 9) Angora cats. The values are shown as mean ± SEM in table.

	Kitten	Adult	Pregnant
Parameters (%)	4.5–6 months	1–3 years of age	15–30 days
$ANAE^+$	68.37a ± 1.22	77.71b ± 1.63	62.89c ± 1.29
$ANAE^-$	31.63a ± 1.22	22.29b ± 1.68	37.11c ± 1.30

nibble fish (9), and Van cats (4). In the present study, optimum results were achieved with a 3 h incubation period at a pH level of 5.8, in contrast to Asti et al. (3) and in agreement with Yoruk et al. (4) for the Van cat. Differences among optimum pH levels reported for the same species are attributed to differences in the age of the animals used in the research, duration of the incubation period, and environmental temperature.

Enzyme cytochemical methods serve as a practical tool in the differentiation of peripheral blood cells and the distinction of tissue T lymphocytes from B lymphocytes. When compared to advanced methods, including immunohistochemical techniques and flow cytometry, ANAE staining is a cheaper and more rapid technique that can be used as a diagnostic method for immunoproliferative or immunosuppressive diseases. Marked alterations in the rate of ANAE positivity of peripheral blood lymphocytes occur with environmental conditions and immune disorders. In particular, significant increases or decreases in the ANAE positivity of lymphocyte rates may occur in viral diseases associated with lymphosuppressive and lymphoproliferative disorders (24).

The reactions that peripheral blood cells produce for ANAE staining also vary. ANAE positivity occurs in different patterns at lymphocytes, monocytes, and macrophages. Several researchers have reported the development of a dot-like or fine granular pattern of positivity in T lymphocytes (9,10). Similar findings have been obtained in the present study. The activity of ANAE, known to give a cytotoxic property to the cell (11), varies among subtypes of T lymphocytes, suggesting that dot-like positivity occurs in cytotoxic T lymphocytes. However, this hypothesis needs to be tested by staining a cytotoxic T lymphocyte cell line grown in culture. On the other hand, B lymphocytes are known to react negatively for ANAE staining (11). In a study conducted by Asti et al. (3), monocytes produced a positive reaction; a diffuse pattern in rats, sheep, and dogs; a strong granular pattern in cats, cattle, and goats; and a weaker granular pattern in horses and chickens. Yoruk et al. (4) reported a diffuse granular positivity in the monocytes and eosinophil granulocytes of the Van cat. In our study, while a diffuse granular positivity was observed in some monocytes, in contrast to the report by Yoruk et al. (4) for the Van cat, eosinophil granulocytes reacted negatively. It is suggested that neutrophil granulocytes react negatively for ANAE staining in cats, dogs, and horses (3). The same literature indicates a negative reaction produced by platelets in cats, horses, cattle, sheep, and goats and by thrombocytes in chickens, while a strong granular positivity is observed in rats and dogs. In the present study, neither granulocytes nor platelets produced a positive reaction for ANAE staining after a 3 h incubation period at pH 5.8.

Previous studies investigating the effect of pregnancy on peripheral blood cells frequently focused on lymphocytes or subtypes. A significant decline is reported in the number of T lymphocytes, B lymphocytes, natural killer cells, and neutrophil granulocytes during early gestation in mares (25), cows (26), and ewes (27). These studies have also revealed an increase in the number of ANAE (+) cells as gestation advances. Çelik et al. (28) reported that ANAE-positive lymphocytes were detected on the 60th day of gestation in the periphery blood circulation of the fetuses, and that the rate of positive lymphocytes increased with the advance of gestation in bovine fetuses. In another study investigating the T lymphocyte rates in the periphery blood circulation of cattle of varying age, Çelik et al. (29) determined that the T lymphocyte rate, which was high during the first few days of life, tended to decline, reaching its peak at 6 to 7 years of age and declining once again at 8 to 9 years of age. Thus, these researchers suggested that T lymphocyte rates follow a fluctuating course in the periphery blood in cattle. Similar findings were obtained in the present study. The rates of ANAE (+) lymphocytes in pregnant, adult, and kitten Angora cats were 62.89 ± 1.29%, 68.37 ± 1.22%, and 77.71 ± 1.63%, respectively. These findings demonstrated that the T lymphocyte rate, which decreased during gestation in the Angora cat, increased over time and reached its peak in adult animals with the development of the immune system.

In the present study ANAE staining performed at a pH level of 5.8 enabled the differentiation of T lymphocytes, which reacted positively, from B lymphocytes. In addition, the number of T lymphocytes differed between pregnant, adult, and kitten Angora cats. These differences were attributed to either the development or the inhibition of the immune system.

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