

Determination of ANAE and ACP-ase Positive Lymphocytes of Peripheral Blood and Endometrium Tissues in Experimental Hypothyroidism-Induced Rats ^[1]

Fatma ÇOLAKOĞLU ¹ Hasan Hüseyin DÖNMEZ ²

^[1] This study was supported by Karamanoglu Mehmetbey University Scientific Research Projects (BAP) Coordinating Office (Project no: 22-M-16)

¹ Department of Nutrition and Dietetics, Faculty of Health Sciences, Karamanoglu Mehmetbey University, TR-70100 Karaman - TURKEY

² Department of Histology and Embryology, Faculty of Veterinary Medicine, Selcuk University, TR-42075 Konya - TURKEY

Article ID: KVFD-2018-20431 Received: 26.06.2018 Accepted: 13.11.2018 Published Online: 14.11.2018

How to Cite This Article

Çolakoğlu F, Dönmez HH: Determination of ANAE and ACP-ase positive lymphocytes of peripheral blood and endometrium tissues in experimental hypothyroidism-induced rats. *Kafkas Univ Vet Fak Derg*, 25 (2): 157-162, 2019. DOI: 10.9775/kvfd.2018.20431

Abstract

This study was aimed to provide information about the status of the immune system by revealing changes in peripheral blood leukocyte (PBL) percentages, ANAE- and ACP-ase(+) lymphocyte rates in peripheral blood (PB) and endometrium tissues of experimental hypothyroidism-induced rats. In this study, 15 healthy female Wistar Albino rats were used. Rats were fed through 4 weeks. The Group E (experimental, n=9) is group that were made hypothyroidism by intraperitoneal methimazole enjection for 2 weeks. Rats of the Group C (control, n=6) were untreated. In the 2nd and 4th weeks, ANAE- and ACP-ase(+) lymphocyte rates of the Group E were higher than Group C in PB. Excepting eosinophil and basophil leukocyte rates, there was no statistical difference in the other PBL percentages in the both of weeks. In PB, while lymphocyte rate of the 4th week was no statistically different (P>0.05), it was found lower in Group C. There was no alteration in ANAE- and ACP-ase(+) lymphocyte rates of uterine tissue. As a result, whereas hypothyroidism caused significant alterations in PBL and T lymphocyte rates, the any marked changes was not observed in the uterine tissue.

Keywords: ACP-ase, ANAE, Endometrium, Hypothyroidism, Methimazole

Deneyel Hipotiroidizm Oluşturulan Ratlardaki Periferik Kan ve Endometriyum Dokularında ANAE ve ACP-az Pozitif Lenfositlerin Belirlenmesi

Öz

Bu çalışma, deneysel hipotiroidi oluşturulan ratların periferik kan lökosit (PKL) yüzdelerindeki, periferik kan ve endometriyum dokularındaki ANAE ve ACP-az (+) lenfosit oranlarındaki değişiklikleri açığa çıkararak bağışıklık sisteminin durumu hakkında bilgi vermeyi amaçlamaktadır. Çalışmada 15 sağlıklı dişi Wistar Albino rat kullanıldı. Ratlar 4 hafta boyunca beslendi. Grup D (deneysel, n=9) 2 hafta boyunca intraperitoneal methimazol enjeksiyonu ile hipotiroidizm oluşturulan gruptur. Grup K'nın (kontrol, n=6) ratları normal beslendi. İkinci ve dördüncü haftalarda, Grup D'nin periferik kandaki ANAE- ve ACP-az(+) lenfosit oranları Grup K'den yüksekti. Eozinofil ve bazofil lökosit oranları hariç olmak üzere, her iki haftada da diğer PKL oranlarında istatistiksel bir fark yoktu. 4. haftadaki periferik kan lenfosit oranı istatistiksel olarak farklı bulunmazken (P>0.05), Grup K'de daha düşük bulundu. Uterus dokusunda ANAE- ve ACP-az(+) lenfosit oranlarında değişiklik görülmedi. Sonuç olarak, hipotiroidizmin PKL ve T lenfosit oranlarında önemli değişikliklere neden olurken uterus dokusunda herhangi belirgin bir değişikliğe yol açmadığı gözlemlendi.

Anahtar sözcükler: ACP-az, ANAE, Endometriyum, Hipotiroidizm, Methimazol

INTRODUCTION

Thyroid hormones play an important role not only in

metabolic disorders ^[1], but also in the development and function of the immune and reproductive systems ^[2].

Hypothyroidism, one of the most common thyroid disorders



İletişim (Correspondence)



+90 338 2262185/4255 GSM: +90 533 3906020



fcolakoglu@kmu.edu.tr

in humans, is insufficient production of thyroid hormones by the thyroid gland [3]. This disorder can be arisen as a consequence of thyroid dysfunction, impediment in mechanism that control thyroid function, or complication during treatment of hyperthyroidism [4]. In studies showing the effects of thyroid hormones on adaptive immunity have been seen that human hypothyroidism, as well as in rodents hypothyroidism induced pharmacologically and surgical, is associated with a decrease in thymic activity. Low concentrations of 3,3',5-triiodo-L-thyronine (T3) and L-thyroxine (T4) can stimulate T cell proliferation including cell-mediated immunity [5,6]. Activation of T lymphocyte subtypes occur in severe hypothyroidism [7]. In clinical cases of hypothyroidism the spontaneous migration of polymorph nuclear leukocytes (PMNL) was found to be impaired [8]. Furthermore, the effects of hypothyroidism are directly on gonadotropin and steroid hormones [9]. Hypothyroidism is one of the most obvious causes of infertility, menstrual disturbance, spontaneous recurrent abortion, and of stillbirths [10]. The effect of hypothyroidism is more marked on the endometrium [11]. One of the most complex tissues is endometrium. Because it undergoes many dynamic changes, such as cytokines, growth factors, hormones and adhesion molecules [12,13]. Martinez et al. [14] reported that T lymphocyte was major class of lymphocytes in uterine.

Alpha-naphthyl acetate esterase (ANAE) demonstration is a method, which is used distinct from each other of T lymphocytes, B lymphocyte and monocytes [15]. Acid phosphatase (ACP-ase) demonstration is also a method and specific for cell populations in which the majority of the T lymphocytes are formed in mammals [16].

This study was planned to provide information about the immune system by revealing changes in peripheral blood leukocyte (PBL) percentages, ANAE- and ACP-ase(+) lymphocyte rates in PB and endometrium tissues of experimentally hypothyroidised rats.

MATERIAL and METHODS

Research Material

Ethic approval was obtained from Selcuk University Experimental Medical Practice and Research Center (SUDAM) Animal Experiments Ethics Committee (2016/13). In this study, it was used 15 healthy female Wistar Albino rats (198-250 g), 12-14 weeks of age. Rats were caged individually on 12:12 h light-dark schedule at the room temperature (22±1°C), and fed with commercial rat food and water which were available *ad bilitum*.

Experimental Procedures

The rats were divided into two groups: animals from the first group (n=9) were made hypothyroid by intraperitoneal (IP) methimazole enjection (10 mg/kg/day) for 2 weeks as

per methods of Parija et al. [17] and Swann [18]. Animals in the other group (n=6) were untreated control (C). In the 2nd week, total serum T3 and T4 concentrations in PB which was taken from the lateral tail veins were determined using the ADVIA Centaur CP Immunoassay System (detection kits provided by Siemens). To see chronic effects on the tissues, the both of groups were made normal feeding for 2 weeks. In the 4th week, the all rats were sacrificed by cervical dislocation under general anesthesia with ketamin (10 mg/kg, IM) and ksilazin (5 mg/kg, IM).

Collection and Processing of Tissue Samples

In the 2nd and 4th weeks, from each blood samples, six blood smears were prepared and fixed in a gluteraldehyde-acetone solution. Two smears were stained for each PBL formula, ANAE and ACP-ase demonstrations [15]. Uterine samples were fixed in formol calcium solution and the samples were incubated in 22 h formal sucrose solution and kept in 22 h Holt's solution for enzyme demonstrations. Then, cryostat sections (12 µm) were taken from samples. These preparations were stained for ANAE and ACP-ase demonstrations [19]. Both PB and uterine tissue samples were stained with 1% methyl green (Merck) for counterstain. For PBL formula, blood smears were stained with May Grünwald-Giemsa staining method [20].

Evaluation of the Stained Tissue Samples

Respectively, as shown in *Fig. 1* and *Fig. 2*, in the PB smears and uterine preparations which were made ANAE demonstration, there were dot-like reddish brown granules of ANAE (+) lymphocytes/T lymphocytes. Respectively, as shown in *Fig. 3* and *Fig. 4*, in PB smears and uterine preparations lymphocytes containing one to three pinkish red cytoplasmic granules were considered to be ACP-ase (+). In each of the PB smears stained for ANAE and ACP-ase activity, 200 lymphocytes were counted and positivity rates were expressed as the percentage of counted cells. For PBL counts, 100 leukocytes were counted in a light microscope, and leukocyte formula were calculated (*Fig. 5*). In the uterine preparations, ANAE- and ACP-ase (+) lymphocytes were counted in the total 0.1 mm² area from 10 different uterine areas.

Statistical Analysis

Comparison of parameters between groups of the 2nd and 4th weeks was analysed using Independent-Samples T test. Differences in parameters between the 2nd and 4th weeks within the groups was drawn using the Paired-Samples T test. Significance was set at P<0.05 [21].

RESULTS

Changes in T3 and T4 levels of groups after methimazole treatment were given *Table 1*. In the 2nd and 4th weeks, T3 and T4 hormone levels were statistically lower in the

Groups	FT3(2)	FT4(2)	FT3(4)	FT4(4)
Control (n=6)	3.22±0.13 ^a	2.35±0.06 ^a	3.35±0.18 ^a	2.39±0.14 ^a
Experimental (n=9)	2.83±0.21 ^b	1.61±0.19 ^b	2.12±0.15 ^b	1.72±0.25 ^b

^{a,b} Values within a column with no common superscripts are significantly (P<0.05) different; FT3(2): 2nd week serum T₃, FT4(2): 2nd week serum T₄, FT3(4): 4th week serum T₃, FT4(4): 4th week serum T₄

Week	FT3	FT4
2 nd	2.99±0.15 ^a	1.90±0.15
4 th	3.27±0.12 ^b	2.00±0.14

^{a,b} Values within a column with no common superscripts are significantly (P<0.05) different

Week	Groups	ANAE(+)	ACP-ase(+)	Lymphocyte	Neutrophil	Monocyte	Eosinophil	Basophil
2 nd	Control (n=6)	21.17±4.68 ^b	37.17±9.25 ^b	80.17±3.71	18.00±4.26	1.17±0.48	0.67±0.21	0.00±0.00
	Experimental (n=9)	74.67±2.52 ^a	49.56±5.81 ^a	73.22±2.85	21.33±3.24	2.11±0.51	1.11±0.39	0.00±0.00
4 th	Control (n=6)	29.17±4.37 ^b	38.50±8.42 ^b	74.17±3.77	21.00±3.70	2.50±0.89	2.00±0.58 ^a	0.33±0.21 ^a
	Experimental (n=9)	68.78±2.20 ^a	47.08±5.68 ^a	78.22±3.53	19.33±3.48	2.33±0.37	0.11±0.11 ^b	0.01±0.00 ^b

^{a,b} Values within a column with no common superscripts are significantly (P<0.05) different

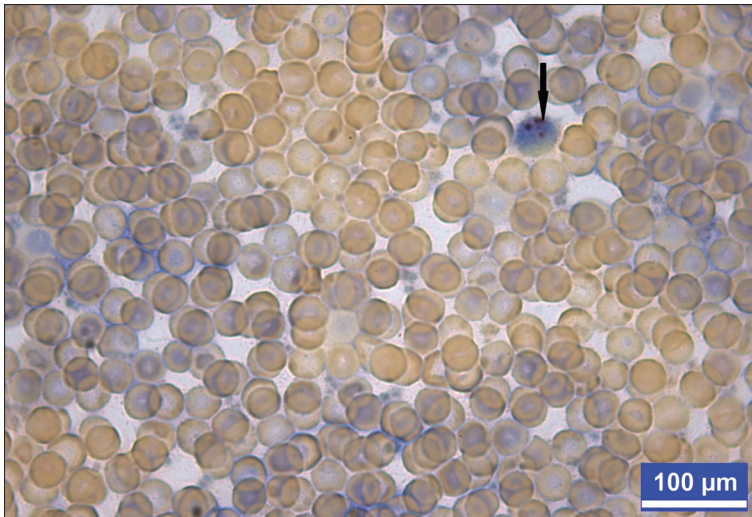


Fig 1. A peripheral blood T-lymphocyte in Group E. ANAE demonstration. Arrow: ANAE (+) lymphocyte/T lymphocyte. Bar: 20 μm

Group E when compared with Group C (P<0.05). According to the weeks within groups, it was statistically seen that T₃ level of 2nd week was lower than that of it in the 4th week (P<0.05) (Table 2). As shown in Table 3, in the 2nd and 4th weeks, the highest peripheral blood ANAE- and ACP-ase (+) lymphocyte (Fig. 1 and Fig. 3, respectively) rates were determined in the Group E (P<0.05). In the 4th week, according to the PBL percentages (Fig. 5), the eosinophil and basophil leukocyte rates were statistically lower in the Group E than that of the Group C (P<0.05). No statistically significant, it was observed that the Group E had higher lymphocyte rate than the Group C

(P>0.05). For both of weeks, there was no statistical difference in the other leukocytes between the groups (P>0.05). No the differences between two weeks within the groups are statistically significant (Table 4). In the endometrium, as shown in Table 5, while the ANAE(+) lymphocyte (Fig. 2) number in the Group C was 19.33/0.1 mm², this rate was found 20.00/0.1 mm² in the Group E. There was no statistical difference between the groups (P>0.05). When the ACP-ase(+) lymphocyte (Fig. 4) number of the Groups C and E were examined, it was noted that there was no statistical difference between the groups (P>0.05).

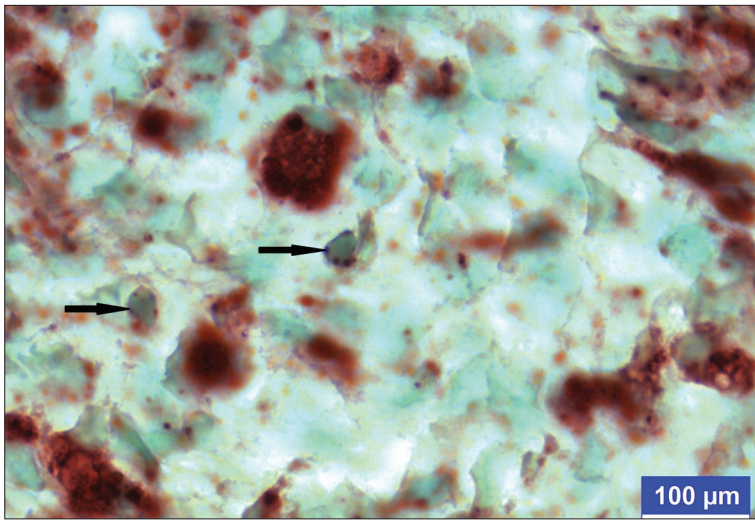


Fig 2. T-lymphocytes in endometrium basalis from Group E. ANAE demonstration. *Arrows:* ANAE (+) lymphocyte/T lymphocyte. Bar: 100 µm

Fig 3. An ACP-ase (+) peripheral blood lymphocyte in a rat from Group E. ACP-ase demonstration. *Arrows:* ACP-ase (+) lymphocytes. Bar: 20 µm

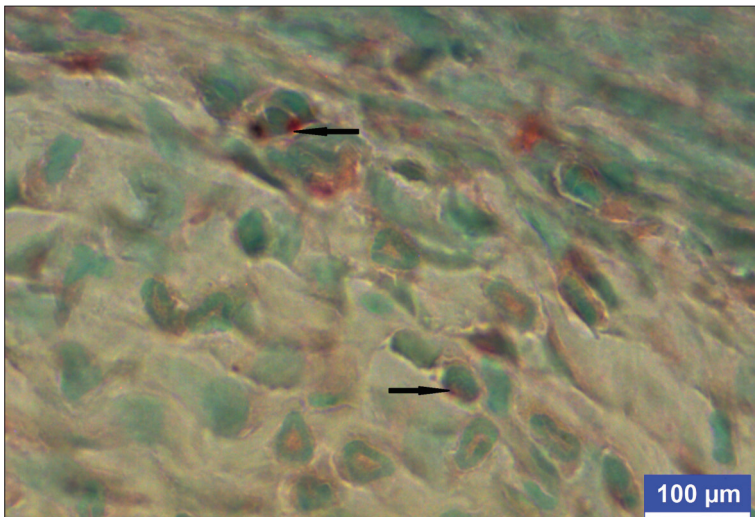
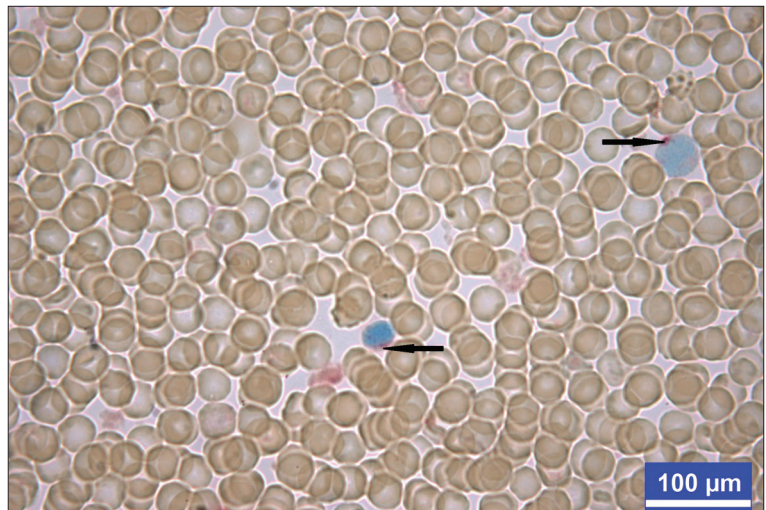


Fig 4. An ACP-ase (+) lymphocyte in endometrium basalis from Group C. ACP-ase demonstration. *Arrows:* ACP-ase (+) lymphocyte. Bar: 100 µm

DISCUSSION

T3 and T4 hormones secreted by the thyroid gland are formed from a large prohormone molecule called as thyroglobulin and enter the cells by binding to the thyroid

hormone receptor α and β [22]. These thyroid hormones which is necessary for sexual development and life regulate the steroid hormones secretion. Findings such as lipidode deficiency, impotence, menopause, excessive bleeding and menstrual irregularities are observed in

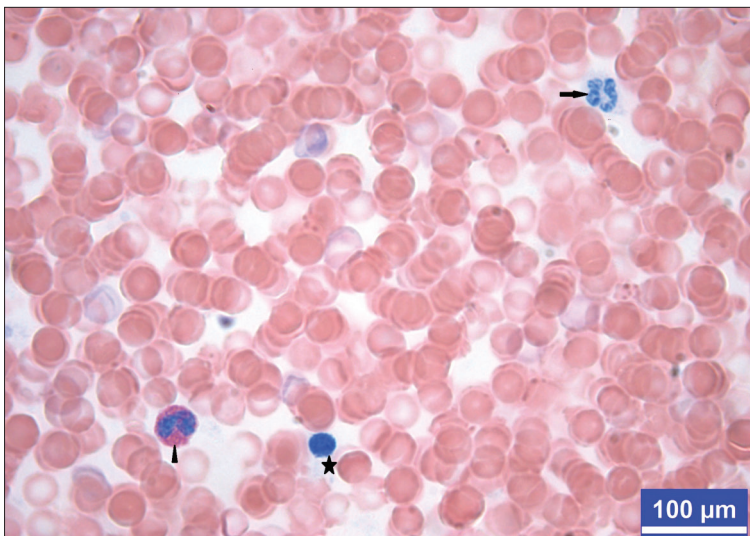


Fig 5. Peripheral blood smear in a rat from Group E. May Grünwald-Giemsa staining. Arrow: Neutrophil, Arrowhead: Eosinophil, Asterisk: Lymphocyte. Bar: 20 μ m

Table 4. Values of parameters between the 2nd and 4th weeks within the groups (%) \pm SE

Week	ANAE(+)	ACP-ase(+)	Lymphocyte	Neutrophil	Monocyte	Eosinophil	Basophil
2 nd	53.27 \pm 7.38	84.60 \pm 12.47	76.00 \pm 2.36	20.00 \pm 2.53	1.73 \pm 0.37	0.93 \pm 0.25	0.00 \pm 0.00
4 th	52.93 \pm 5.59	89.60 \pm 4.61	76.60 \pm 2.57	20.00 \pm 2.48	2.40 \pm 0.40	0.87 \pm 0.34	0.13 \pm 0.91

No the differences between two weeks within the groups are statistically significant ($P>0.05$)

Table 5. The proportions of the ANAE(+) ve ACP-ase (+) lymphocytes in endometrium basalis region of uterine (%) \pm SE (number/0.1 mm²)

Week	Groups	ANAE(+)	ACP-ase(+)
4 th	Control (n=6)	19.33 \pm 0.80	8.50 \pm 0.62
	Experimental (n=9)	20.00 \pm 0.71	8.00 \pm 0.44

No the differences between the groups are statistically significant ($P>0.05$)

hypothyroidism. Although the most synthesized hormone in the thyroid gland is T4, the most effective is the T3 [23].

In humans, one of the most common thyroid disorders is hypothyroidism, which is defined as low levels of thyroid hormones in the blood [3]. Deficiency of thyroid hormone production causes serious abnormalities [1]. For establishing hypothyroidism in experimental animals, methimazole is frequently used in the treatment of human hyperthyroidism [24]. In this study, the methimazole dose and form of implementation given to the Group E was determined from the results of previous studies [17,18].

ANAE is an enzyme that demonstrates T lymphocytes. It is also known that dot-like positivity is specific for T lymphocytes [15]. ACP-ase is one of the lysosomal enzymes in lymphocytes. Some investigators have demonstrated ACP-ase reactivity in human peripheral blood T lymphocytes [15,16]. In the direction of this information, we found that T lymphocyte and ACP-ase (+) lymphocyte rates were higher in the Group E ($P<0.05$). This situation was explained that low serum T3 and T4 concentrations can stimulate T lymphocyte proliferation [7].

Although we did not statistically find any change in the other PBL excluding eosinophil and basophil leukocytes, we was observed that the lymphocyte ratio was higher in the Group E. This suggests that thyroid hormones have regulatory effects on immunological activity at the cellular level [25]. There is also information that thyroid hormones modify lymphocyte activity [26,27]. In severe hypothyroidism, activation of T lymphocyte subclasses, reduction of natural killer cells and reduction of CD4 T lymphocyte responses are seen [7]. Compared with healthy subjects, hypothyroidism has been found to be impaired in the spontaneous migrations of polymorphonuclear leukocytes in clinical cases [8]. Hypothyroidism is accompanied by spleen and lymph node involution and decreases in humoral and cellular immune responses [28].

As in other systemic mucosal tissues, uterine mucosa normally contains T and B lymphocytes [29]. However, the uterine tissue under the influence of hormonal changes is rearranged with the sexual cycle or the pregnancy. Karaca et al. [30] reported that the distribution of ANAE (+) T lymphocyte numbers in uterine tissues of pre-implantation period of goats was lower than that of non-pregnant animals. Akbulut et al. [15] reported significant reductions in ANAE- and ACP-ase (+) lymphocyte numbers throughout the entire pregnancy in the decidua basalis region of endometrium. In the direction of the data obtained from this study, there was no change in terms of both ANAE- and ACP-ase (+) lymphocyte numbers and distribution in endometrium basalis. We can say that hypothyroidism has no any an effect on uterine in terms of lymphocyte number and distribution.

Prevention of mental and developmental disorders is possible with a good understanding of the interaction between the endocrine and immune systems. The immune system is under the influences of many hormones. Routine control and treatment of thyroid gland functions at pre-pregnancy and throughout pregnancy is an important factor for a healthy pregnancy. As a result of this study, hypothyroidism made significant changes both the peripheral blood T lymphocyte and leukocytes counts. Obtained findings may help doctors to evaluate the immunological status of hypothyroid women. Since these techniques are simple, much cheaper, less time consuming applications, we suggest that it can be given as a laboratory service to assist of women in the early diagnosis of some gestational disorders. However, further studies should be planned in terms of our understanding more detailed of relationship between thyroid gland diseases and uterine.

As a result, whereas hypothyroidism caused significant alterations in PBL and T lymphocyte rates, the any marked changes was not observed in the uterine tissue.

ACKNOWLEDGEMENT

We thank to "Karamanoglu Mehmetbey University Scientific Research Projects (BAP) Coordinating Office", Project no: 22-M-16, for financial support.

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