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Relationship Between Serum Carbonic Anhydrase Activity and Carbonic Anhydrase Autoantibody Levels in Patients with Rheumatoid Arthritis

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Abstract

Objective*: An organism's immune response to its own tissue antigens is called autoimmunity, and the diseases caused by such, autoimmune diseases. Carbonic anhydrase II, (CA II), the most common CA isoenzyme in humans, is considered a common target antigen, since it is abundantly synthesized in bile ducts, pancreatic ducts, renal tubular cells, and the epithelial cells of salivary glands, in addition to erythrocytes. Therefore, anti-CA measurements have become important in the diagnosis of some autoimmune diseases.*

Method:*The effect of the carbonic anhydrase enzyme in the measurement of CA I and CA II antibodies using Enzyme-Linked Immunosorbent Assay (ELISA) was investigated using the serum of a group of rheumatoid arthritis patients.By this aim, total CA amount was found via determination of the amounts of CA I and CA II antibodies and of esterase activity by ELISA method.*

Results: *CA II autoantibody levels in the patient group of 0.255 ±¬ 0.182 (mean + SD), and 0.137 ± 0.040 in the control group were found ($p < 0.001$). Meanwhile, CA I autoantibody level was found to be 0.153 ± 0.036 in the patient group, and the control group level was 0.129 ± 0.052; ($p = 0.965$). There was no correlation between CA I/CA II autoantibody levels and serum CA activity.*

Conclusion*: Thus, it was concluded that the determination of CA antibodies by ELISA method is not affected by CA activity in non-hemolytic serum.*

Keywords: *carbonic anhydrase, autoimmunity, rheumatoid arthritis, hemolysis*

1. Introduction

Carbonic anhydrase (CA), (CA, EC 4.2.1.1., carbonate hydrolyase) is an enzyme found in many species, ranging from bacteria to the most developed animals. These enzymes are metalloproteins containing Zn^{2+} ions in their active regions and are in monomer structure. Carbonic anhydrases play a role in physiological events, such as the transport of CO_2 formed in the organism by metabolic pathways, electrolyte secretion, regulation of the acid-base balance, and the formation of biosynthetic HCO_3^- (Menteşe et al., 2018). To date, 16 different CA isoenzymes have been identified in different cells and tissues of animals, with various cellular locations (Menteşe et al., 2017a).

Autoimmune diseases are described as the body itself produces (auto)antibodies against its own healthy tissue antigens (Menteşe et al., 2017b). In 1991, the presence of CA II autoantibodies was identified for the first time in the serum of patients with systemic lupus erythematous and Sjogren's syndrome (Inagaki et al., 1991). Thereafter, CA II autoantibodies in various autoimmune diseases were determined using western blot and Enzyme-Linked Immunosorbent Assay (ELISA) methods (Kino-Ohsaki et al 1996, .Ito et al. 1997, Gordon et al.,1995). CA II is synthesized abundantly in the epithelial cells of bile ducts, pancreatic ducts, renal tubules, and salivary gland ducts. Therefore, CA II is thought to play a role in the pathogenesis of disease complexes called autoimmune exocrinopathy and autoimmune epithelitis, which are secreted from epithelial cells of various exocrine glands (Nishimori et al. 2004). In addition to these, CA II autoantibodies have been identified in patients with systemic lupus erythematous, systemic sclerosis, dermatomyositis, polymyositis, endometriosis, autoimmune hepatitis and chronic viral

hepatitis, in end-stage renal disease, acute anterior uveitis and PCOS. However, the role of CA II autoantibodies in the pathology of the abovementioned diseases is not understood (Invernizzi et al., 1998, Akisawa et al, 1999, Alver et al. 2014, Turk et al., 2014, Menteşe et al., 2013,).

Autoimmune diseases such as endometriosis, autoimmune thyroid, rheumatoid arthritis, systemic sclerosis, idiopathic chronic pancreatitis, and Sjögren's syndrome affect about 5% of the population in Western countries (Menteşe et al., 2018). In recent years, it has been discussed whether CA II antibodies can be used as serum markers in identifying some of these autoimmune diseases and distinguishing them from diseases with similar symptoms (Aparisi et al., 2005). None of the previous studies have examined the possible effect of CA activity, which may be caused by hemolysis, on the autoantibody measurements.

Although blood collection methods are standardized, there is carbonic anhydrase activity present in serum and carbonic anhydrase activity from erythrocytes resulting from minimal hemolysis. Aim of this study is to reveal the quantitative relationship between total CA activity and CA I and II autoantibody measurements in rheumatoid arthritis patients' sera and whether these values correlate with total CA activity originating from hemolysis.

2. Materials and Methods

Sample Collection

For the study, permission was obtained from the clinical research ethics committee of Karadeniz Technical University KTU) with a meeting number of 04/2007 and decision

number 2007/8, and the patients were informed with an informed consent form. Sera used in this study were obtained from rheumatoid arthritis patients (n = 32, 25 female/7 male) who applied to the KTU Faculty of Medicine Physical Medicine and Rehabilitation outpatient clinic, and the blood used for the control group was taken from volunteer individuals (n = 24, 9 female/15 male). Collected blood samples were centrifuged at 3000 rpm for 10 minutes, and the sera obtained were stored at -20°C.

Enzyme-Linked Immunosorbent Assay

Determination of the CA I and CA II autoantibody levels was performed using the ELISA method developed before, with minor modifications (Hosoda et al., 2004). Each sample was assayed in duplicate and the specific binding of serum antibody to CA I or II was calculated with the following formula :

Specific binding = ODcoated – ODcontrol.

Determination of Carbonic Anhydrase Activity

Determination of CA activity in serum was performed using the esterase activity measurement method developed by Armstrong et al. (Verpoorte et al., 1967). The method is based on the hydrolysis of p-nitro phenyl acetate to *p*-nitro phenol and acetate by carbonic anhydrase. Carbonic anhydrase activity against p-nitro phenyl acetate was determined by measuring the amount of released p-nitro phenol spectrophotometrically at 348 nm.

To determine CA activity in serum samples, at the first glance, total esterase activity was determined in each serum. For this purpose, 1.3 mL Tris-SO_4 buffer was well mixed with 0.1 mL serum or 0.1 mL buffer solution at room temperature. Then, on top of this mixture,

1.5 mL of *p*-nitro phenyl acetate solution was added, and its absorbance was read at 348 nm immediately. After 3 min of incubation time, the absorbance was read again for the same samples. The difference between the absorbance values in the first and second read was calculated as esterase activity. With this experiment, the overall activities of total CA and other ester hydrolysis enzymes in serum were measured.

In the second part, CA in serum was completely inhibited using 0.1 M acetazolamide solution. 1.3 mL Tris-sulfate buffer added to test tubes with 0.1 mL serum and 0.1 mL inhibitor solution. This mixture was incubated for ten minutes to ensure full enzyme inhibitory interaction. Then, 1.5 mL of substrate solution was added to it, and absorbance was read at 348 nm immediately. Following this, samples were incubated for three minutes, absorbance was read again, and differences between two time points was calculated. By subtracting total esterase activity found in the first experiment from the value in the second part of the experiment, the CA activity within the total esterase activity was determined. Absorbance values were used for statistical analysis.

Statistics

Fitness of the data obtained from the patient and control groups to normal distribution was determined by the Kolmogorov-Smirnov test. Student's t test was used for those that conformed to the normal distribution, and Mann-Whitney U test was used for those that did not fit the normal distribution. Significance was considered at the level of $p < 0.05$.

3. Results

ELISA Results for CA I Autoantibodies

The levels of CA I autoantibody were analyzed using ELISA method in serum collected from

32 rheumatoid arthritis patients and 24 healthy individuals. Individuals with an average absorbance value greater than 0.233 were considered positive (cut off value). This value was calculated by adding 2SD (standard deviation) value (0.052) to the average absorbance value (0.129) in the control group. While none of the 32 patients with rheumatoid arthritis had positive results, one positive result was found in the control group (4.2%). (Figure 1)

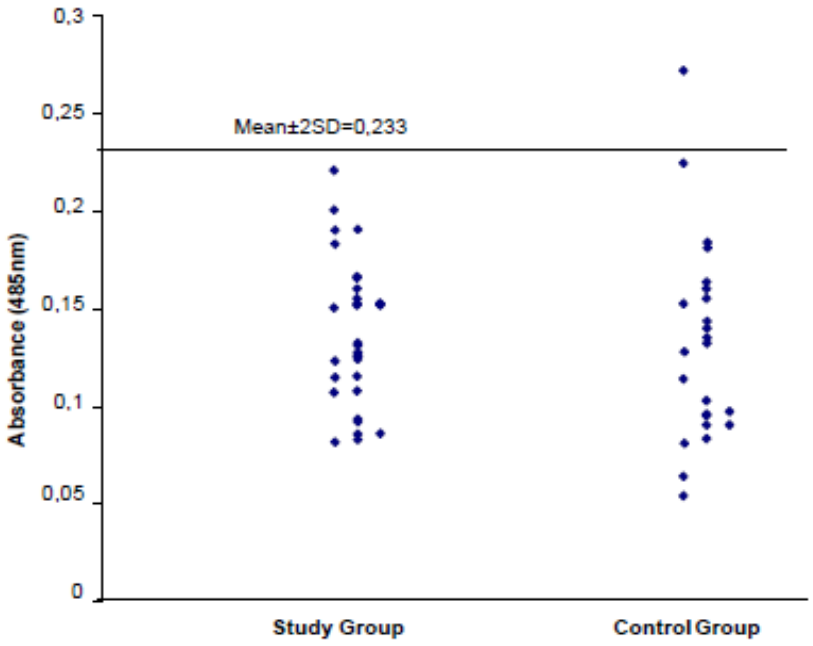

Figure 1. ELISA Results for CA I Autoantibodies

ELISA Results for CA II Autoantibodies

The sera of 32 patients with rheumatoid arthritis and 24 healthy individuals were evaluated for CA II autoantibody levels by ELISA method. Individuals with absorbance values greater than 0.217 were considered positive. This value was calculated by adding 2SD (0.040) to the average absorbance level of the control group. On 17 of the patients

with rheumatoid arthritis, the sera was found positive (53%). No positive results were found in the control group. (Figure 2)

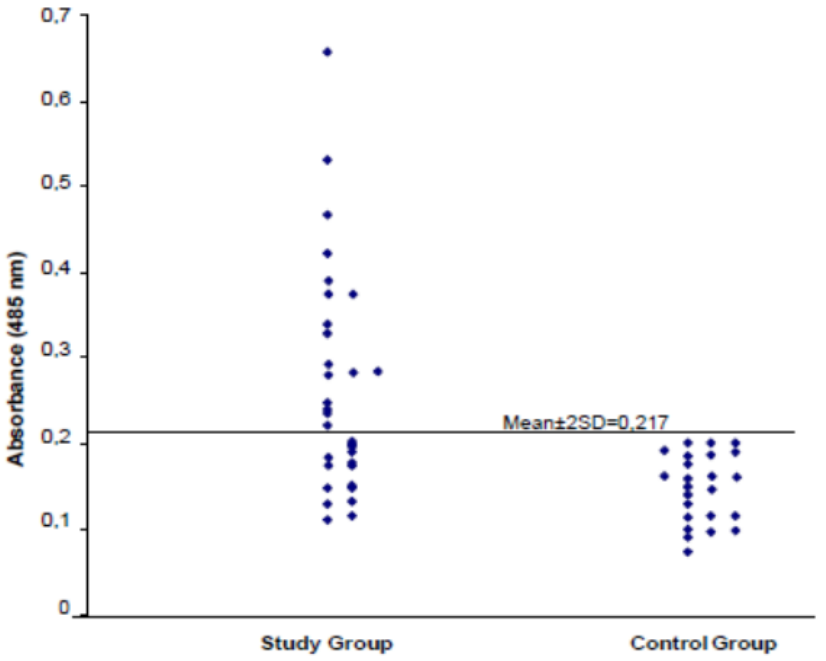

Figure 2. ELISA Results for CA II Autoantibodies

Statistical Results

CA II autoantibody level in the patient group was 0.255±0.128 (mean+SD) and 0.137±0.040 in the control group, and there was a statistically significant difference between them ($p<0.001$).CA I autoantibody level in the patient group was 0.135 ±0.036, and 0.129 in the control group.It was found ±0.052, and there was no statistically significant difference between them ($p=0.965$).(Table 1)

Table 1. Statistical analysis of ELISA for CA I and CA II antibodies

	Patient (n = 32)	Control (n = 24)
CAI autoantibody	0.135 ± 0.036	0.129 ± 0.052
CAII autoantibody	0.255 ± 0.128*	0.137 ± 0.040

** Mann Whitney U test, p<0.001*

Pearson correlation test was used to determine whether there was a relationship between CA I and CA II autoantibody levels and esterase activity. Here, the activity of CA is taken as absorbance and not as enzyme unit, and these values are also not given in tabular form. There was a very low positive correlation between CA I autoantibody level and esterase activity in the patient group (r=0.016), but it was not statistically significant (p=0.927). There was a low negative correlation between CA I autoantibody level and esterase activity in the control group (r=-0.157), but it was not found to be statistically significant (p=0.435). A low and insignificant negative correlation (r=-0.269, p=0.107) was obtained between CA II autoantibody values and esterase activities in the patient group. In the control group, a low negative correlation (r=-0.026) and a statistically insignificant (p=0.898) correlation was found between CA II autoantibody level and esterase activity.

4. Discussion

After Inagaki et al. showed the presence of carbonic anhydrase enzyme in systemic lupus erythematous and Sjogren's syndrome, CA II autoantibodies were detected in different autoimmune diseases using western blot and ELISA methods (Kino-Ohsaki et al 1996, .Ito et al. 1997, Gordon et al.,1995). CA II is synthesized abundantly in the epithelial cells of bile ducts, pancreatic ducts, renal tubules, and salivary gland ducts. Therefore, CA II is

thought to play a role in the pathogenesis of disease complexes called autoimmune exocrinopathy and autoimmune epithelitis as a common target antigen secreted from epithelial cells of various exocrine glands (Nishimori et al., 2004).

The main task of the immune system is to recognize foreign antigens and create an immune response against them. B and T lymphocytes and macrophages are responsible for the initiation and control of immune response (Menteşe et al., 2017b). Under physiologic conditions, organisms do not produce immunity against their own antigens. However, as a result of some pathologic conditions, malfunction of receptors and/or changed/delayed activities of immune system members, the organism can exhibit autoimmunity.

In the diagnosis of autoimmune diseases with unknown mechanism, the determination of cellular type of immune responses and of autoantibodies against the organism's own tissues is used.

Autoimmune diseases, such as endometriosis, autoimmune thyroiditis, rheumatoid arthritis, systemic sclerosis, idiopathic chronic pancreatitis, and Sjögren's syndrome affect about 5% of the population in western countries (Menteşe et al., 2018). Rheumatoid arthritis manifests as a nonspecific and generally symmetrical inflammation in peripheral joints. It is a chronic inflammatory disease that is likely to result in progressive destruction in and around the joints. In recent years, the use of CA II autoantibodies as a serum marker has been discussed in determining and identifying some of these autoimmune diseases (Akisawa et al.,1999, Aparisi et al., 2005,). Previously, in our department, studies were performed based on determination of CA II antibodies by ELISA method. (Alver et al., 2014,

Turk et al., 2014, Menteşe et al., 2013, Menteşe et al., 2015, Menteşe et al,. 2017c). Effect of hemolysishad not been investigated so our results will contribute the literature. For this purpose, in the serum of 32 patients with rheumatoid arthritis, together with 24 controls, total CA activities and CA I and II antibodies were measured, and the relationship between them was quantitatively examined.

As a result of the ELISA tests, while all the data for anti-CA I measurement was negative, 53% of anti-CA II measurements were positive. In ELISA measurements made in different disease groups, anti-CA I levels were found to be greater and more positive than anti-CA II (Kino-Ohsaki et al.,1996, Bartolome et al.,2002, Frulloni et al.,2000). However, our measurements gave totally opposite results, and there was no significant difference between the control and patient groups. As a result, it can be claimed that anti-CA I levels do not change in rheumatoid arthritis.

Carbonic anhydrase enzymes can be found in serum as a result of hemolysis of erythrocytes. With this study, we wanted to demonstrate whether the carbonic anhydrase enzymes affect the ELISA measurements of anti-CAs in hemolytic situations. For this purpose, we performed a correlation test between total CA and anti-CA activities measured in patient and control groups. As a result, in the patient group, a low positive ($r = 0.016$) and statistically insignificant ($p = 0.927$) correlation between CA I autoantibody level and esterase activity was calculated. In the control group, a negative ($r = -0.157$) and statistically insignificant ($p = 0.435$) correlation between CA I autoantibody level and esterase activity was found. Similarly, a negative ($r = -0.269$) and statistically insignificant ($p = 0.107$) correlation was observed between CA II autoantibody level and esterase

activity in the patient group. In the control group, a negative (r =-0.026) and statistically insignificant (p = 0.889) correlation was found between CA II autoantibody level and esterase activity. It was concluded that unless there is an easily noticeable excessive hemolysis, it is not necessary to consider serum CA activity in serum ELISA measurements.

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Conflict of Interest

The authors stated that there were no conflicts of interest.

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