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Diagnostic Value of Thrombocyte Aggregates in Sarcoidosis by Flow Cytometric Analysis

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Abstract
Sarcoidosis is a granulomatous disease characterized by a multisystemic, hyperimmune response that can frequently involve the lung and lymphatic system, but has the potential to involve all organs in the body. A type IV hypersensitivity reaction, in which T cells play a role, is seen with cellular immune response. In addition to clinical and radiological findings, non-caseous granulation seen in biopsy material is used in its diagnosis. In addition, it has been shown that the CD4/CD8 ratio in the bronchoalveolar lavage fluid can be used together with other parameters in the diagnosis of the disease. In this study, it was aimed to evaluate the role of the presence of aggregates in monocyte-neutrophil cell lines in the diagnosis of the disease and the usability of the CD4/CD8 ratio in peripheral blood in patients diagnosed with sarcoidosis.
15 patients with sarcoidosis and 15 healthy individuals were included in the study. Analysis of the samples was carried out in a flow cytometry device (BD, FacsCalibur) using dyes suitable for lymphocyte surface markers CD4-8 and platelet surface markers CD41a-61-14.
In the study, no difference was found between CD4/CD8 ratios. In the monocytic series, there was a significant difference between the patient and control groups in terms of CD14-CD61($p<0.001$), CD41a($p=0.045$) and CD61($p<0.001$) values. In addition, a significant difference was found in CD41a ($p=0.035$) and CD61 ($p<0.001$) values in neutrophils when comparing patient and control groups ($p<0.05$).
By using the data of the study, further studies are planned that are thought to contribute to the parameters that can be used in the diagnosis of sarcoidosis.

Keyword: Sarcoidosis, Flow cytometry, CD4/CD8, CD41, CD61

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Sarkoidozda Akım Sitometrik Analizle Trombosit Agregatlarının Tanısal Değeri

Özet
Sarkoidoz sıklıkla akciğer ve lenfatik sistemi tutabilen ancak vücutta tüm organlarda da tutulum yapma potansiyeli olan, multisistemik, hiperimmün yanıtla karakterize granülomatöz bir hastalıktır. Hücresel immun yanıtla T hücrelerin rol oynadığı tip IV hipersensitivite reaksiyonu görülür. Tanısında, klinik ve radyolojik bulguların yanı sıra biyopsi materyalinde görülen non-kazeöz granülasyon kullanılmaktadır. Ayrıca bronkoalveoler lavaj sıvısındaki CD4/CD8 oranının hastalığın tanısında diğer parametreler ile birlikte kullanılabileceği gösterilmiştir. Bu çalışmada, sarkoidoz tanısı almış hastalarda, monosit-nötrofil hücre serilerinde agregatların varlığının, hastalık tanısındaki yerinin ve CD4/CD8 oranının periferik kanda da kullanılabilirliğinin değerlendirilmesi amaçlandı.
Çalışmaya, sarkoidoz tanılı 15 hasta ve 15 sağlıklı birey alındı. Örneklerin analizinde lenfosit yüzey belirteçlerinden CD4/8, trombosit yüzey belirteçlerinden CD41a-61-14 için uygun boyalar kullanılarak, akım sitometri cihazında (BD,FacsCalibur) gerçekleştirildi.
Çalışmada, CD4/CD8 oranları arasında fark olmadığı bulundu. Monositer seride, CD14-CD61($p<0,001$), CD41a($p=0,045$) ve CD61($p<0,001$) değerleri bakımından hasta ve kontrol grupları arasında anlamlı farklılık olduğu tespit edildi. Ayrıca, hasta ve kontrol grupları karşılaştırmasında nötrofillerde, CD41a ($p=0,035$) ve CD61 ($p<0,001$) değerleri bakımından anlamlı farklılık olduğu bulundu ($p<0,05$).
Yapılan çalışmanın verilerinden yararlanılarak, sarkoidoz tanısında kullanılabilecek parametrelere katkı sağlayacağı düşünülen ileri çalışmalar planlanmaktadır.

Anahtar kelime: Sarkoidoz, Flow sitometri, CD4/CD8, CD41, CD61

Introduction

Sarcoidosis is a multisystemic, granulomatous disease characterized by a hyperimmune response and it can frequently involve the lung and lymphatic system, but has the potential to involve all organs in the body.[1] Type IV hypersensitivity reaction, in which T cells play a role in the cellular immune response, is seen. Mononuclear cells consisting of CD4+ helper T-lymphocytes (Th) and monocyte-macrophages are collected in the involved organs. In this process, a large number of released cytokines and chemokines play a role, and as a result, granuloma formation occurs. There is a lymphocyte cell group consisting of CD4+ T-lymphocyte, lesser number of CD8+ T-lymphocyte and B-lymphocyte around the granuloma and there are mononuclear phagocytes, epithelioid and multinuclear giant cells in the center of it. Therefore, the CD4+/CD8+ ratio increases due to the increase in the number of CD4+ T cells in areas with granulomas. Since most of the cases have lung involvement, CD4+/CD8+ ratio increases in bronchoalveolar lavage (BAL). It has also been shown that the CD4/CD8 ratio in the bronchoalveolar lavage fluid can be used together with other parameters in the diagnosis of the disease [2,3].

In patients with thrombosis, 20-30% of initial venous thromboembolic events have been shown to be Ca-related. Leukocytes and thrombocytes play an important role in the formation of thrombosis. In various clinical situations, neutrophil/lymphocyte (NLR) and platelet/lymphocyte (PLR) ratios are increased in malignancies, respiratory diseases, cardiovascular diseases, and many inflammatory diseases, and it has been shown that high levels are associated with the poor prognosis and low levels are associated with the good prognosis.[4]
Neutrophils play a role in the pathogenesis of many important inflammatory diseases. Neutrophils perform their antimicrobial activities by regulating tissue factor production and thrombocyte activation through oxygen radicals.[5] Thrombocytes support tissue infiltration in the inflammation region and adhesion of leukocytes to the endothelium by extravasation of leukocytes. Cells trigger monocytes and endothelial cells to acquire a procoagulant phenotype by secreting inflammatory cytokines.[6]

P-selectin is expressed by activated thrombocytes and endothelial cells and is used as a marker of thrombocyte activation. P-selectin in activated thrombocytes initiates interaction with leukocytes and monocytes, triggers the inflammatory cascade, and stimulates the formation of leukocyte-thrombocyte aggregates. There are data showing that LTA is increased in conditions such as DM, stroke, and MI, which are associated with the increased prothrombotic tendency.[6]
In this study, it was aimed to evaluate the role of the presence of aggregates in monocyte-neutrophil cell lines and the usability of the CD4/CD8 ratio in the peripheral blood in the diagnosis of sarcoidosis.

Material and Method
Peripheral blood samples of 15 patients and 15 healthy individuals diagnosed with sarcoidosis by Mersin University Faculty of Medicine, Department of Chest Diseases between November 2019 and January 2020 were included in the study. Venous whole blood of the patients was used in this study. Blood samples were taken into 2 mL tubes containing ethylenediaminetetraacetic acid, and the samples were studied within 8 hours.
For immunophenotype analyzes of T cells and monocyte-neutrophil aggregation, CD3 (BD Biosciences, USA), CD4 (BD Biosciences, USA), CD8 (BD Biosciences, USA), CD14 (BD Biosciences, USA), CD61 (BD Biosciences, USA) and the frequency and number of CD41a (BD Biosciences, USA) were measured by flow cytometry using the FACSCalibur (Becton Dickinson, San Jose, CA, USA) device.
The manufacturer's recommended amount of antibody was added to a 5 mL polyethylene tube. 100 µl of whole blood with EDTA was added to the tube and incubated for 15 minutes at room temperature in the dark. Red blood cells (RBC) were lysed with the RBC Lysis Solution kit (BD Biosciences, USA) based on the manufacturer's recommended protocol. It was centrifuged at 1,300 rpm for 5 minutes. The upper supernatant was removed with a pipette. The precipitate was washed with 2 ml of PBS. Then 500 µl of PBS was added to the precipitate. Flow cytometric measurement was performed on the FACSCalibur instrument (Becton Dickinson, USA). 20,000 events were counted for each sample, and results were analyzed using the CellQuest Pro program. The lymphoid region was selected to determine the frequency and number of T cells (CD3, CD4, CD8). Both monocytes and neutrophils were used in order to determine the frequency and number of CD14, CD41a and CD61.

Statistical analysis
The normal distribution control of the study variables in each group was evaluated with the Shapiro-Wilk normality test. Group comparisons of variables found to be incompatible with normal distribution in at least one group were evaluated with the non-parametric Mann-Whitney U test. Percentages (25-75%) and median values were given as summary statistics. Group comparisons of the variables compatible with the normal distribution were evaluated with the parametric method, two independent groups t-tests. Mean, standard deviation, minimum and maximum values were given as summary statistics.

Results
There was no difference between the mean age of the 15 patients with sarcoidosis and the control group consisting of 15 individuals ($p=0.871$). Parametric Independent two-group t-tests results were evaluated after Levene variance homogeneity control.
The mean values of WBC, %MONO, MPV, CD3, CD8, CD41a (neutrophil), CD61 (neutrophil) parameters in the patient group were determined as 8.13, 8.50, 10.55, 73.04, 29.793, 19.91 and 20.36, respectively. The mean values in the control group were WBC 6.85, %MONO 7.33, MPV 10.64, CD3 74,740, CD8 28,120, CD41a (neutrophil) 14.55 and CD61 (neutrophil) 10.6733. While there was a statistically significant difference between the patient and control groups in terms of CD41a (neutrophil) and CD61 (neutrophil) values ($p<0.05$), there was no significant difference in terms of WBC, %MONO, MPV, CD3 and CD8 values (Table 1A). %LYMPH, %NEUT, %EO, %BASO, CD4, CD4/CD8, CD14-CD61 (monocyte), CD41a (monocyte) and CD61 (monocyte) variables were not found compatible with normal distribution ($p<0.05$) Nonparametric Mann Whitney-U test used. A statistically significant difference was found between the patient and control groups in terms of CD14-CD61 (monocytes), CD41a (monocytes) and CD61 (monocytes) values ($p<0.05$). However, no significant difference was found in terms of %LYMPH, %NEUT, %EO, %BASO, CD4 and CD4/CD8 values (Table 1B).

Table 1. Results of complete blood count and flow cytometry analysis of the study groups

1A.

Group		Age	WBC	%MONO	MPV	CD3	CD8	CD41a (neutrophil)	CD61 (neutrophil)
Patient	**Mean**	56.13	8.13	8.50	10.55	73.04	29.79	19.91	20.36
	SD	11.10	2.21	2.276	0.82	11.57	11.27	8.32	7.17
	Min	29	5.39	4	9.40	53.5	10.6	3.9	11.30
	Max	79	12.44	13	11.90	89.7	46.8	36.9	37.40
Control	**Mean**	56.80	6.85	7.33	10.64	74.74	28.12	14.55	10.67
	SD	10.14	1.14	1.718	0.79	8.71	7.33	4.3119	3.87
	Min	40	4.62	5	9.40	59.0	17.0	8.0	5.60
	Max	69	9.26	12	12.20	87.1	42.9	21.0	20.10
p		**0.871**	**0.075**	**0.141**	**0.774**	**0.653**	**0.634**	**0.035**	**<0.001**

WBC: White Blood Cell; Mono: Monocyte; MPV: Mean Platelet Volume; SD:Std. Deviation; Min: Minimum; Max: Maximum

1B.

Group		%LYMPH	%NEUT	%EO	%BASO	CD4	CD4/CD8	CD14-CD61 (monocyte)	CD41a	CD61
Patient	**Q1**	23.00	50.75	0.70	0.33	32.80	0.83	12.20	16.60	16.50
	Median	32.00	57.00	2.25	0.95	40.50	1.42	20.50	21.00	25.20
	Q3	34.50	62.00	4.1	1.00	51.20	2.45	25.50	28.40	27.50
Control	**Q1**	26.00	52.00	1.60	0.60	35.40	1.27	3.60	10.40	6.10
	Median	29.00	60.00	1.90	0.70	41.60	1.60	5.60	16.00	8.40
	Q3	35.00	65.00	2.40	0.80	48.00	1.86	7.40	21.20	10.20
p		**0.964**	**0.427**	**0.981**	**0.516**	**0.713**	**0.806**	**<0.001**	**0.045**	**<0.001**

LYMPH: Lymphocyte; NEUT: neutrophils; EO: eosinophil; BASO: *basophil; Q1* (Lower Quartile) = 25%; *Median* = 50%; Q3 (Upper Quartile) = 75%.

Discussion

It is known that the CD4/CD8 ratio is important for sarcoidosis patients. There are many studies on how the CD4/CD8 ratio changes in especially BAL fluid. However, there are not enough studies in the literature to explain whether the determination of this ratio in whole blood contributes to the diagnosis of the disease.

According to the study of Marjolein Drent et al. in 2007, there was not a single cell presented in BAL fluid in the diagnosis of sarcoidosis. The cell types and numbers in the BAL fluid help in the diagnosis and exclusion of many diseases from sarcoidosis to interstitial lung diseases. In individual cases, on the other hand, the CD4/CD8 ratio is less important, as this ratio may show an increasing or decreasing

trend or remain at normal levels. In this study, the researchers concluded that this ratio was not very important in the treatment and prognosis follow-up of the disease.[7]
In another study conducted in 2009, they investigated the role of CD4/CD8 ratio in BAL in the diagnosis of different radiological and clinical forms of pulmonary sarcoidosis. Researchers have reported that BAL fluid can be used as a different method in the diagnosis of sarcoidosis, but the CD4/CD8 ratio in BAL fluid shows high variability, and accordingly, its role in the diagnosis of the disease continues to be controversial, and this ratio may vary depending on clinical cases and radiographic findings.[8]
Since sarcoidosis and tuberculosis cause granulomatous inflammation, it is not always possible to differentiate with pathological samples. For this reason, it is argued that there is a need for new markers that can be used to distinguish these diseases. In a retrospective study conducted in 2014, the use of NLR in the differential diagnosis of these two diseases was investigated, and it was reported that NLR levels were found to be significantly higher in patients with tuberculosis compared to those with sarcoidosis, and it could be a useful marker in the differentiation of diseases.[9]
In a retrospective study on the levels of platelet/lymphocyte ratios of patients with sarcoidosis, it was reported that there was a significant increase in PLR levels in correlation with the involvement of the lung parenchyma in patients with sarcoidosis. [10]
It is thought that NLR and PLR studies with hematological parameters will provide a great advantage in the diagnosis of sarcoidosis, which is mainly manifested by lung involvement. In 2020, in a study by Korkmaz et al., it was found that in sarcoidosis patients, PLR and NLR levels were weakly positively correlated with CRP, while there was a moderate positive correlation between NLR levels and blood CD4/CD8 levels. In addition, a very strong positive correlation was found between CD4/CD8 and NLR levels in BAL fluid, while a moderate positive correlation was reported with PLR. As a result, it has been argued that high PLR and NLR may not always be associated with the prognosis, response to treatment and spontaneous remission.[11]
In this study, it was determined that there was a significant increase in NLR levels in patients with sarcoidosis. In addition, PLR levels were found to be elevated in sarcoidosis patients. There was no difference between the patient and control groups in terms of CD4/CD8 ratios. In the monocytic series, a significant difference was found between the patient and control groups in terms of CD14-CD61 ($p<0.001$), CD41a ($p=0.045$), and CD61 ($p<0.001$) values. In addition, a significant difference was found in CD41a ($p=0.035$) and CD61 ($p<0.001$) values in neutrophils when comparing patient and control groups ($p<0.05$).
There are studies on the CD4/CD8 ratio in sarcoidosis and bronchoalveolar lavage fluid. However, it should be noted that the variability seen in the data of these studies may be due to differences in disease distributions in populations or to other dominant diseases such as tuberculosis in specific populations or geographic regions. In this study, peripheral blood was used as an example, and it is thought that when the data obtained are supported by further studies, it can contribute to the parameters that can be used in the diagnosis of sarcoidosis.

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