Original Article

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Investigation of Anti-neutrophil Cytoplasmic Antibody by Indirect Immunofluorescence Assay in COVID-19 Patients

Zeynep Ayaydin*, Nida Ozcan**, Cigdem Mermutluoglu***, Selahattin Atmaca**, Ali Cem Tekin****

*Mardin Artuklu University Faculty of Medicine, Department of Medical Microbiology, Mardin, Turkey

**Dicle University Faculty of Medicine, Department of Medical Microbiology, Diyarbakir, Turkey

***Dicle University Faculty of Medicine, Department of Infectious Diseases and Clinical Microbiology, Diyarbakir, Turkey

****University of Health Sciences Turkey, Gazi Yasargil Training and Research Hospital, Department of Medical Microbiology, Diyarbakir, Turkey

Abstract

Aim: Coronavirus disease-2019 (COVID-19) has been suggested to trigger the production of autoimmune antibodies and contribute to the development of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. This study aims to investigate the presence of ANCA among COVID-19 patients.

Methods: This cross-sectional, prospective analysis included 200 COVID-19 patients with positive polymerase chain reaction test results for severe acute respiratory syndrome-coronavirus-2 and no history of autoimmune disease, recruited between June 2021 and November 2022. The control group included 50 age-matched healthy blood donors. The ANCA profile was assessed using the indirect immunofluorescence assay method with the EUROPLUS Granulocyte Mosaic EUROPattern test kit (EUROIMMUN, Germany) on sera samples of the patient and control groups.

Results: Perinuclear ANCA (p-ANCA) was detected in 12 of 200 COVID-19 patients (6.0%) and cytoplasmic ANCA (c-ANCA) was detected in 15 of 200 patients (7.5%). No ANCA positivity was observed in the control group (0/50). ANCA positivity among COVID-19 patients (27 of 200, 13.5%) was statistically significantly higher than in the control group ($p<0.05$). ANCA positivity was significantly higher in intensive care unit (ICU) patients (21 of 77, 27.3%) compared to non-ICU patients (6 of 123, 4.9%) ($p<0.05$).

Conclusion: ANCA presence in ICU patients supports the hypothesis that COVID-19 triggers ANCA synthesis and contributes to disease severity.

Keywords: COVID-19, anti-neutrophil cytoplasmic antibodies, vasculitis, indirect immunofluorescence assay

Introduction

In addition to specific antibodies in coronavirus disease-2019 (COVID-19) patients, researchers have investigated whether autoantibodies are produced that can increase the severity of the disease by attacking some cells and tissues. Although the exact reason for the formation of autoantibodies is not known, two different theories have been proposed. First, the development of a hypersensitivity reaction within the immune system due to the coronavirus infection, which can damage the patient's cells and tissues. Second, some of the virus-specific antigenic epitopes are similar to the patient's cell antigens (1,2).

Corresponding Author: Zeynep Ayaydin
Mardin Artuklu University Faculty of Medicine, Department of Medical Microbiology, Mardin, Turkey
E-mail: zeynepayaydin@gmail.com **ORCID:** orcid.org/0000-0002-4701-1212
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Autoantibodies formed in COVID-19 are recognized as one of the key factors influencing disease severity. It has been reported that antinuclear antibody, antiphospholipid antibody, anti-type 1 interferon antibody, and, rarely, anti-neutrophil cytoplasmic antibody (ANCA) are encountered frequently (40-50%) in COVID-19 cases. The number of original studies investigating ANCA levels in COVID-19 patients is very low. With these limited studies, research indicates that ANCA production is triggered in some cases. ANCA, whose production can be triggered in COVID-19, can cause serious organ damage, leading to vasculitis development in the respiratory tract, kidneys, and small arteries in the skin. In the COVID-19 pandemic, cases of immune glomerulonephritis have been reported as a result of vasculitis due to ANCA production (3-5). We hypothesized that COVID-19 is associated with vasculitis and that autoantibodies capable of causing vasculitis could be detected in patients with COVID-19.

The primary aim of this study is to investigate the presence of ANCA, which may be triggered in COVID-19 patients, and to highlight the risk of vasculitis that ANCA could induce. Thus, the detection of ANCA autoantibodies in COVID-19 patients in clinical practice will contribute to predicting disease progression, taking preventive measures, and developing treatment protocols. The data obtained from this study will serve as a resource for more comprehensive research to be conducted in the future.

Methods

Compliance with Ethical Standards

This study was approved by the decision dated 25.02.2021 and approval numbered 233 of the Dicle University Faculty of Medicine Ethics Committee for Non-Interventional Studies. Informed consent forms were obtained from the patients and control group members.

Study Design

The study was a cross-sectional analysis conducted between June 2021 and November 2022. A total of 200 patients, aged 18-90 years, who presented to the Dicle University Hospital COVID-19 outpatient clinic or were hospitalized in the COVID-19 clinic or intensive care unit (ICU) with a positive polymerase chain reaction (PCR) test and no prior history of autoimmune disease were included. The age, gender, and clinical information of the patients who were followed up/treated were recorded. Fifty healthy blood donors who did not have COVID-19, did not have a positive PCR test, and had no known autoimmune disease were selected as the control group.

Venous blood samples (5-6 mL) were collected from the patients and the control group under aseptic conditions and delivered to the indirect immunofluorescence assay (IFA) laboratory within 30 minutes. After the blood was centrifuged, the serum was separated and kept in a deep freezer at -80 °C until it was studied.

One of the methods used to investigate the ANCA profile is the IFA. In our study, the EUROPLUS Granulocyte Mosaic EUROPattern (EUROIMMUN, Germany) IFA test kit was used to investigate the ANCA profile. Positive and negative controls were included for each run.

The patient's diluted blood serum was dripped onto the slides in the commercial kit, and the preparation was allowed to sit in accordance with the procedure recommended by the manufacturer. After the first wash with the buffer solution, the anti-human antibody (conjugate) labeled with fluorescein isothiocyanate was added, and then the second wash was performed with the buffer solution, waiting for an appropriate time. Finally, the slides were covered with glycerol and mounted with a coverslip to be examined under a fluorescent microscope. Slides were examined under a fluorescent microscope as soon as possible.

The ethanol-fixed chamber was examined and cytoplasmic/perinuclear differentiation of ANCA was performed. Cytoplasmic ANCA (c-ANCA) positivity: a diffuse, granular green fluorescent uptake is observed in the cytoplasm into the neutrophil cytoplasm, while there is no fluorescent uptake in the cell nucleus. In perinuclear ANCA (p-ANCA) positivity, there is green fluorescent uptake surrounding the neutrophil nucleus (6).

The formalin-fixed chamber is then examined. In formalin-resistant ANCA types, only fluorescent staining is seen in the cytoplasm. However, fluorescent uptake is not seen in formalin-sensitive ANCA types. In formalin-resistant c-ANCA types, positive fluorescence is mostly observed in the proteinase 3 (PR3) compartment, whereas in formalin-resistant p-ANCA types, positive fluorescence is mostly observed in the myeloperoxidase (MPO) compartment.

Blood serum with non-fluorescent neutrophils was recorded as ANCA negative. In the formalin- and ethanol-fixed chamber, granular-looking fluorescent positivity was evenly distributed in the cytoplasm of neutrophils, and the samples showing green circular fluorescence in the PR3 area were recorded as c-ANCA, PR3 formalin-resistant positive. C-ANCA appeared in the ethanol-fixed chamber, but this fluorescence disappeared in the formalin-fixed chamber. Samples that did not fluoresce in PR3 were recorded as c-ANCA, formalin-sensitive PR3 negative.

Patients with perinuclear fluorescence surrounding the fragmented nuclei of neutrophils in the ethanol-fixed chamber, fluorescence scattered in the cytoplasm in the area with formalin, and circular green fluorescence in the MPO area were recorded as p-ANCA, formalin-resistant MPO positive. Samples that are formalin-sensitive but do not fluoresce in this area and MPO were recorded as p-ANCA, while formalin-sensitive MPO was recorded as negative.

Statistical Analysis

The obtained data were analyzed using SPSS version 20.0. Percentages were reported for categorical variables to reflect the distribution of the data. Chi-square tests were performed to assess relationships between categorical variables (gender, age, and unit of care). If the expected frequency was less than 20% in any cell, the p-value from Fisher's exact test was reported instead of that from the chi-square test. In statistical analysis, a 95% confidence interval was used, and a p-value of <0.05 was considered statistically significant.

Results

P-ANCA was positive in 12 (6.0%) patients and c-ANCA in 15 (7.5%) patients out of 200 in the patient group, while ANCA was not detected in the control group (n=50). Eleven patients who tested p-ANCA positive were formalin sensitive/MPO negative, and one patient was formalin resistant/MPO positive, while all c-ANCA positive patients were formalin sensitive/PR3 negative. The distribution of ANCA positivity according to the COVID-19 patient and control groups is presented in Table 1. The positivity rate in the patient group was high (13.5%), and the difference compared to the control group was statistically significant (χ^2=8.291, p=0.004).

Four (3.6%) out of 112 female patients and eight (10.0%) out of 88 male patients were found to be p-ANCA positive. Seven (6.3%) of 112 female patients and eight (10.0%) of 88 male patients were found to be c-ANCA positive. The difference in p-ANCA positivity and gender (χ^2=2.648, p=0.104) and c-ANCA positivity and gender (χ^2=0.605, p=0.437) in the patient group is not statistically significant. There is no difference between the two genders.

The distribution of p-ANCA positivity and c-ANCA positivity according to the clinic where the patients are followed/treated is presented in Table 2 and Table 3, respectively. The differences between p-ANCA positivity (χ^2=6.269, p<0.05) and c-ANCA positivity (χ^2=16.295, p<0.05) and the clinic where the patients were followed/treated were statistically significant. It is noteworthy that both p-ANCA and c-ANCA positivity are significantly higher in patients hospitalized in the COVID-19 intensive care unit than in patients hospitalized in the general or outpatient clinic.

The distribution of p-ANCA positivity by age group is presented in Table 4, while c-ANCA positivity is in Table 5. It was observed that both p-ANCA and c-ANCA positivity rates were higher in patients over 60 years of age. However, the difference between p-ANCA positivity and age was statistically significant (χ^2=5.404, p=0.020), while the difference between c-ANCA positivity and age was not significant (χ^2=2.698, p=0.100).

Table 1. Distribution of ANCA positivity by COVID-19 patient and control groups

Group name	ANCA		χ2	p-value
	Positive/n (%)	Negative/n (%)		
Patient group	27 (13.5)	173 (86.5)	8.291	0.004
Control group	0	50 (100.0)		

Fisher's exact test was performed
COVID-19: Coronavirus disease-2019, ANCA: Anti-neutrophil cytoplasmic antibody

Discussion

Autoantibodies are rarely encountered in COVID-19 patients. ANCA-related vasculitis is a condition that may develop in COVID-19 patients. It is an inflammatory process that damages small arteries in the body, including the respiratory system, kidneys, and skin (1,2,5).

Table 2. Distribution of p-ANCA positivity according to the clinic where the patients were followed/treated

The patient's	p-ANCA		χ2	p-value
	Positive/n (%)	Negative/n (%)		
Intensive care unit	8 (10.4)	69 (89.6)	6.269	0.044
Clinic	4 (6.1)	62 (93.9)		
Outpatient clinic	0	57 (100.0)		
Total	12 (6.0)	188 (94.0)		

Fisher's exact test was performed
p-ANCA: Perinuclear anti-neutrophil cytoplasmic antibody

Table 3. Distribution of c-ANCA positivity according to the clinic where the patients were followed/treated

The patient's	c-ANCA		χ2	p-value
	Positive/n (%)	Negative/n (%)		
Intensive care unit	13 (16.9)	64 (83.1)	16.295	0.00
Clinic	2 (3.0)	64 (97.0)		
Outpatient clinic	0	57 (100.0)		
Total	15 (7.5)	185 (92.5)		

Fisher's exact test was performed
c-ANCA: Cytoplasmic anti-neutrophil cytoplasmic antibody

Table 4. Distribution of p-ANCA positivity by age

Age	p-ANCA		χ2	p-value
	Positive/n (%)	Negative/n (%)		
≤60	1 (1.3)	77 (98.7)	5.404	0.020
>60	11 (9.0)	111 (91.0)		
Total	12 (6.0)	188 (94.0)		

Fisher's exact test was performed
p-ANCA: Perinuclear anti-neutrophil cytoplasmic antibody

Table 5. Distribution of c-ANCA positivity by age				
Age	c-ANCA		χ2	p-value
	Positive/n (%)	Negative/n (%)		
≤60	3 (3.9)	75 (96.1)	2.698	0.100
>60	12 (9.8)	110 (90.2)		
Total	15 (7.5)	185 (92.5)		
Fisher's exact test was performed c-ANCA: Cytoplasmic anti-neutrophil cytoplasmic antibody				

In many studies conducted during the pandemic and in the following years, rapidly progressive necrotizing glomerulonephritis cases have been reported due to ANCA positivity-related vasculitis in patients with COVID-19 (7-15). In addition, cases that were reported in this period of ANCA-associated pediatric vasculitis that developed after COVID-19 were noted (16,17).

The studies presented above show that there may be an association between ANCA production, triggered by severe acute respiratory syndrome-coronavirus-2 infection, and vasculitis, as well as organ damage, that may develop. It is also thought that autoantibodies increase the severity of the disease and prolong the recovery period. On the other hand, it has been determined that autoantibodies formed during the disease can remain in the body for a long time afterward (18-20). Recent studies have indicated that COVID-19 may trigger the production of ANCA, leading to the development or exacerbation of vasculitis. Therefore, assessing patients for autoantibody positivity during or after the acute phase of COVID-19 infection is crucial for evaluating disease progression and optimizing the treatment protocol. Similarly, some studies conducted recently have demonstrated the development of ANCA-associated vasculitis following COVID-19 vaccination, as well as after COVID-19 infection (21-23).

In the literature, there are two other studies, with 45 and 80 patients respectively, that have investigated the presence of ANCA autoantibodies in COVID-19 patients using a methodology similar to that of this study. However, the number of patients included in this study (200 patients) is significantly higher than that of the other studies (24,25).

Study Limitations

The limitations of this study include the limited number of patients, inability to obtain repeated samples, and the exclusion of specific ANCA antibodies other than MPO and PR3. Despite these limitations, the detection of ANCA positivity in COVID-19 patients, particularly those in the intensive care unit, clearly demonstrates the laboratory association between COVID-19 and vasculitis. The number of cases in this study is still higher than similar studies in the literature (e.g., 45 and 80 cases) (24,25).

Conclusion

We think that COVID-19 may trigger autoimmune diseases, including ANCA-related vasculitis. Thus, we demonstrated through laboratory data that COVID-19 infection triggers ANCA production, leading to vasculitis that exacerbates disease severity. This finding highlights the clinical significance of the study. In the literature, the number of original studies on the development of ANCA-related vasculitis and ANCA positivity in COVID-19 patients is very low. Therefore, more extensive research on the subject is needed. We believe that these study data will be a source for more comprehensive research.

Ethics

Ethics Committee Approval: This study was approved by the Non-Interventional Studies Ethics Committee of the Dicle University Faculty of Medicine (date: 25.02.2021, approval no.: 233).

Informed Consent: Informed consent forms were obtained from the patients and control group.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Z.A., N.O., A.C.T., Concept: Z.A., N.O., C.M., S.A., Design: Z.A., N.O., Data Collection or Processing: C.M., A.C.T., Analysis or Interpretation: A.C.T., Literature Search: Z.A., N.O., C.M., S.A., Writing: Z.A.

Conflict of Interest: No conflicts of interest were declared by the authors.

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