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Antinuclear antibodies detected by Enzyme-Linked Immunosorbent Assay (ELISA) in severe COVID-19: clinical and laboratory associations

M. BRIANZA-PADILLA[1], Y. JUÁREZ-VICUÑA[1], R. SPRINGALL[1], J. GONZÁLEZ-FLORES[1], M. PATLÁN[2], L.M. AMEZCUA-GUERRA[1,3]

[1]Immunology Department, [2]Basic Research Subdirectorate, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City, Mexico
[3]Health Care Department, Universidad Autónoma Metropolitana Xochimilco, Mexico City, Mexico

Abstract. – OBJECTIVE: Antinuclear antibodies (ANA) are detected in approximately a quarter of COVID-19 patients when assessed by indirect immunofluorescence. Since there is no information, our study investigated the presence of ANA detected by Enzyme-Linked Immunosorbent Assay (ELISA) and its clinical and laboratory associations.

PATIENTS AND METHODS: A longitudinal study was conducted on 92 patients with severe COVID-19, 20 patients with acute myocardial infarction, and 25 healthy subjects. Blood samples were obtained at hospital admission. Commercial ELISA was used to detect ANA, while flow cytometry was used to measure serum interferons.

RESULTS: ANAs were positive in 8.6% of COVID-19 patients, 10% of myocardial infarction patients, and 4% in healthy individuals (p=0.676). COVID-19 patients with ANA+ had less ferritin, troponin, and neutrophils but more albumin and lymphocytes than ANA– patients. Serum levels of type I, II, and III interferons were similar between groups. At follow-up, all ANA+ patients survived, while mortality was significant in ANA– patients (0 *vs.* 36%; p=0.048).

CONCLUSIONS: ANA detection is not increased in severe cases of COVID-19 when assessed by ELISA. However, its presence appears to be associated with a less aggressive disease phenotype, regardless of circulating levels of interferons.

Key Words:
Antinuclear antibodies, COVID-19, ELISA, Interferons.

Introduction

The COVID-19 pandemic continues despite vaccination, social isolation, and the use of masks around the world. An immunological diathesis resembling systemic autoimmunity with cytokine storm, coagulopathy, and acute tissue injury, is found as part of the devastating manifestations of SARS-CoV-2 infection. Further linking hyper inflammation to autoimmunity, antinuclear antibodies (ANA) are detected by indirect immunofluorescence (IIF) in approximately a quarter of COVID-19 patients[1-5]. Despite being limited by poor standardization and lack of optimal cut-off points, while depending on operator subjectivity and experience, IIF remains the gold standard for ANA detection, due to its high sensitivity[6,7]. Other detection platforms may remedy some of these shortcomings. For example, the enzyme-linked immunosorbent assay (ELISA) is a fully standardized and automated platform that uses well-characterized recombinant antigens as a substrate, thus balancing the specificity and sensitivity of the assay[7].

Since there is no information about the presence of ANA evaluated by ELISA, our study investigated these antibodies at hospital admission of patients with severe COVID-19 and their clinical and laboratory associations.

Patients and Methods

Patients

Patients admitted to respiratory triage from April to July 2020 for severe COVID-19 with a positive test (RT-PCR in nasopharyngeal swab) for SARS-CoV-2 were recruited. Inclusion criteria: age > 18 years, CT signs of pneumonia, and at least one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or oxygen saturation < 90% on room air. Exclusion criteria: history of any autoimmune disease or malignancy, positive rapid influenza test, or use of medications known to cause positive ANA.

Corresponding Author: Luis M. Amezcua-Guerra, MD, Ph.D; e-mail: lmamezcuag@gmail.com

All patients gave their consent to use their clinical data for research purposes. Approval was granted by the local Ethics Committee (No. 20-1186), and the procedures were carried out following the 2013 Declaration of Helsinki, its addenda, and local regulations.

Laboratory Procedures

Sera were obtained at hospital admission for routine measurement of inflammatory and thrombotic markers. ANAs were detected using the ANA Detect ELISA kit (Orgentec Diagnostika, Germany), which qualitatively detects IgG antibodies against centromere B, histone proteins H1, H2A, H2B, H3, H4, histidyl-tRNA synthetase, mononucleosomes, Pm-Scl-100, polynucleosomes, RNP-A, RNP-C, RNP-70, RNP/Sm complex, DNA topoisomerase I, Ro52, Ro60, La, ssDNA, histone complex, Sm-BB, Sm-D, Sm-E, Sm-F, Sm-G or dsDNA. Stored sera from 20 patients with acute myocardial infarction (AMI) and 25 healthy individuals were analyzed as reference. Serum levels of type I (IFN-α2, IFN-β), II (IFN-γ), and III (IFN-λ1, IFN-λ2/3) interferons were measured with the LEGENDplex Human 1/2/3 Interferon Panel (BioLegend, San Diego, CA, USA) on a BD FACSAria Fusion flow cytometer.

Statistical Analysis

Frequencies and percentages were used to describe categorical data, and differences were analyzed using Fisher's exact or chi-square tests. Continuous variables were expressed as means ± standard deviation or medians with interquartile range (IQR) and were compared using the Mann-Whitney *U* test. All analyzes were 2-tailed, and $p<0.05$ was set for significance. GraphPad Prism v.9 (GraphPad Software, La Jolla, CA, USA) was used for calculations.

Results

92 COVID-19 patients (67% male; age 56.1 ± 12.7 years) were included (Table I). 8 COVID-19 patients were positive for ANA (8.6%), compared with two AMI patients (10%) and only one healthy individual (4%; $p=0.676$ for all comparisons).

When we analyzed COVID-19 patients according to their ANA status (Table I), we found a higher proportion of women among the ANA+ patients (75% *vs.* 29%; $p=0.013$). In addition, ANA+ patients had lower leukocytes and neutrophils and less ferritin and troponin I than their ANA– counterparts. A higher lymphocyte count and higher albumin levels were found in ANA+ patients. Sera from 5 of 8 ANA+ and 60 of 84 ANA– patients were available for interferon measurement. Notably, there were no significant differences in the levels of type I, II, or III interferons (Table I).

At follow-up, case fatality in the COVID-19 cohort reached 33%. Although there were no differences in the therapies and interventions administered or in the occurrence of thrombosis, all ANA+ patients survived, while mortality was substantial in ANA-patients (0 *vs.* 36%; $p=0.048$). There was also no difference in length of hospital stay.

Discussion

Previous studies showed higher than expected ANA positivity in COVID-19 when assessed by IIF. Pascolini et al[1] reported that 11 of 33 patients had ANA+ in low titers, compared to 25 non-COVID-19 controls (33% *vs.* 8%; $p=0.02$). The frequency of ANA was not different between the survivors and those who ultimately died (27% *vs.* 57%; $p>0.10$). Chang et al[2] reported that 10 of 47 patients (21%) were ANA+, although the titers were mostly weak (mean dilution 1:40). The mortality rate between ANA+ and ANA– patients was similar (10% *vs.* 8%). In the study by Sacchi et al[3], 23 of 40 patients were positive compared to only 5 of 40 healthy subjects (57% *vs.* 12%; $p<0.001$). Interestingly, 9 of 23 ANA+ patients and 2 of 17 ANA– patients ultimately died (39% *vs.* 11%; $p=0.07$). Finally, a study[4] from Greece found that 10 of 29 (34%) patients were ANA+, while another study from Italy[5] described 16 of 45 (35%) patients with CT-proven pneumonia as ANA+. Both studies reported no clinical or serological associations based on ANA status.

The advent of solid-phase immunoassays containing recombinant antigens allows for a more specific laboratory test that detects antigen-specific antibodies rather than naturally occurring ANA, as IIF does[8]. Lerma et al[9] detected ANA+ in 16 of 64 (25%) COVID-19 patients by multiplexed bead-based assay. Of the reactive samples, 75% were from critically ill patients receiving advanced life support care. However, ANAs were weakly reactive and targeted to single antigens, as is often seen during acute infection. In our study, ANA detection was performed by ELISA, which can reveal the presence of serum IgG antibodies

Table I. Clinical and laboratory data of COVID-19 patients.

	All (n=92)	ANA positive (n=8)	ANA negative (n=84)	p
Age (years), mean ± SD	56.1 ± 12.7	56.2 ± 14.4	56.1 ± 12.6	0.970
Male, n (%)	62 (67)	2 (25)	60 (71)	**0.013**
BMI (kg/m^2), mean ± SD	28.9 ± 4.9	28.1 ± 3.5	28.9 ± 5.0	0.765
Hypertension, n (%)	42 (45)	4 (50)	38 (45)	0.990
Diabetes, n (%)	40 (43)	1 (12)	39 (46)	0.131
Vital signs on hospital admission				
• SaO_2 at room air, mean ± SD	78.0 ± 13.6	83.8 ± 5.8	77.4 ± 14.0	0.314
• Heart rate, mean ± SD	97.2 ± 19.9	93.5 ± 13.6	97.6 ± 20.4	0.409
• Respiratory rate, mean ± SD	25.2 ± 6.4	22.5 ± 7.8	25.5 ± 6.2	0.064
• Temperature (° C), mean ± SD	37.0 ± 0.8	36.9 ± 0.6	37.0 ± 0.8	0.792
Laboratory measurements on admission				
• Leukocytes, 1x10^3/μL	10.3 (7.1-13.3)	5.5 (4.5-7.6)	10.8 (7.5 -13.6)	**0.006**
• Neutrophils, 1x10^3/μL	8.8 (6.1-12.0)	3.8 (3.2-6.2)	9.2 (6.5-12.1)	**0.003**
• Lymphocytes, 1x10^3/μL	0.8 (0.5-1.1)	1.2 (0.8-1.5)	0.8 (0.5-1.0)	**0.046**
• NLR	10.5 (6.5-18)	3.7 (2.8-4.9)	10.8 (7.3-19.6)	**<0.001**
• Hemoglobin, g/dL	14.8 (13.3-16.1)	14.5 (13.6-14.6)	14.9 (13.2-16.2)	0.192
• Platelets, 1x10^3/μL	225 (173-288)	187 (152-300)	228 (179-287)	0.497
• D-dimer, ng/mL	457 (246-846)	301 (150-566)	488 (258-891)	0.070
• Ferritin, μg/L	657 (351-1,103)	258 (200-328)	674 (416-1,196)	**0.004**
• Albumin, g/dL	3.4 (3.1-3.7)	3.6 (3.5-3.9)	3.3 (3.0-3.7)	**0.038**
• Troponin I, ng/mL	18.3 (6.3-76)	5.1 (3.6-5.6)	20.6 (8.3-91.5)	**<0.001**
• Interleukin-6, pg/mL	55.3 (4.5-89.1)	21.2 (4.5-60.7)	16.2 (4.5-89.1)	0.710
• C-reactive protein, mg/L	158 (74-259)	93 (68-193)	167 (87-260)	0.209
• IFN-α2, pg/mL	5.4 (0.9-13.4)	5.8 (0.9-16.1)	5.4 (0.9-13.6)	0.913
• IFN-β, pg/mL	3,170 (77-4,458)	3,961 (1,630-5,011)	3,084 (45-4,438)	0.335
• IFN-γ, pg/mL	1,964 (14-2,518)	2,152 (1,018-2,525)	1,740 (14-2,527)	0.692
• IFN-λ1, pg/mL	2,802 (133-6,392)	4,371 (1,863-5,756)	2,722 (133-6,428)	0.913
• IFN-λ2/3, pg/mL	542 (103-830)	795 (306-1,067)	541 (53-819)	0.351
Major clinical outcomes				
• Vascular thrombosis, n (%)	21 (22)	1 (12)	20 (23)	0.677
• Mechanical ventilation, n (%)	53 (57)	2 (25)	51 (60)	0.067
• Death, n (%)	31 (33)	0	31 (36)	**0.048**
Days of hospital stay	14 (10-26)	9 (7-14)	14 (11-26)	0.096

ANA: antinuclear antibodies; BMI: body mass index; IFN: interferon; NLR: neutrophil-to-lymphocyte ratio; SD: standard deviation. Data are presented as median (interquartile range) unless otherwise specified. Significant *p*-values are in bold.

directed only against the antigens contained in the plaque. This high specificity may explain the similar frequency of ANA+ by ELISA in COVID-19 patients compared to those with acute tissue injury (AMI) and healthy individuals, which is significantly lower than that observed when detected by IIF.

The other highlight of the present study is the impact that ANA status has on the clinical course of COVID-19. Previous studies[4,10] have suggested that ANA+ patients may develop severe disease and higher mortality, while others[2,3] have shown no significant associations. Our study included the largest number of patients in a trial designed specifically to detect ANA in COVID-19. ANA positivity was associated with attenuated inflammation and less tissue and endothelial damage. Furthermore, all deaths occurred in patients who were negative for ANA. We speculate that interferon levels could underlie our findings, as other coronavirus infections of concern, such as SARS-CoV-1 and MERS-CoV, require an intense interferon response to remove viral particles and facilitate tissue repair[10]. As described above, no differences in interferon response between ANA+ and ANA– patients were noted.

Our findings are provocative despite discrepancies with previous reports. Alternative explanations should be considered, including different methods for detecting ANA, cut-off points for defining ANA positivity in IIF, heterogeneity in the severity of COVID-19, and the ethnicity of each population studied. Our main limitation is the lack of simultaneous measurement of ANA by IIF. The strengths of

this study are a close hospital follow-up, the number of patients studied, and the assessment of novel inflammatory mediators, namely interferons. Whether ANAs detected by ELISA have predictive value in real-world clinical settings remains to be elucidated.

Conclusions

Although ANA detection is not increased in severe cases of COVID-19 when assessed by ELISA, its presence appears to be associated with a less aggressive disease phenotype, regardless of circulating levels of interferons.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

Approval was granted by the local Ethics Committee (Comité de Ética en Investigación del Instituto Nacional de Cardiología Ignacio Chávez; protocol number 20-1186).

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