Italy), L.S. (Università Vita-Salute San Raffaele and IRCC Ospedale San Raffaele, Milan, Italy), Vittorio Stefoni (University of Bologna, Bologna, Italy), L.T. (University of Padua, Padua, Italy), and M.V. (Fondazione IRCCS Policlinico San Matteo, Pavia, Italy).

REFERENCES

1. Foà R, Bonifacio M, Chiaretti S, et al. Philadelphia-positive acute lymphoblastic leukaemia (ALL) in Italy during the COVID-19 pandemic: a Campus ALL study. *Br J Haematol*. 2020;190(1):e3-e5.
2. Li W, Wang D, Guo J, et al. COVID-19 in persons with chronic myeloid leukaemia [published online ahead of print 18 May 2020]. *Leukemia*. doi: 10.1038/s41375-020-0853-6
3. Breccia M, Abruzzese E, Bocchia M, et al. Chronic myeloid leukemia management at the time of the COVID-19 pandemic in Italy. A Campus CML survey [published online ahead of print 18 June 2020]. *Leukemia*. doi: 10.1038/s41375-020-0904-z
4. Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol*. 2019;94(11):1266-1287.
5. Wierda WG, Byrd JC, Abramson JS, et al. Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma, Version 4.2020, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2020;18(2):185-217.
6. Treon SP, Castillo JJ, Skarbnik AP, et al. The BTK inhibitor ibrutinib may protect against pulmonary injury in COVID-19-infected patients. *Blood*. 2020;135(21):1912-1915.

DOI 10.1182/blood.2020006854

TO THE EDITOR:

Red cell–bound antibodies and transfusion requirements in hospitalized patients with COVID-19

Alessandra Berzuini,[1,*] Cristiana Bianco,[1,*] Cinzia Paccapelo,[1] Francesco Bertolini,[2] Giuliana Gregato,[2] Alessandra Cattaneo,[1] Elisa Erba,[1] Alessandra Bandera,[1,3] Andrea Gori,[1,3] Giuseppe Lamorte,[1] Maria Manunta,[1] Laura Porretti,[1] Nicoletta Revelli,[1] Francesca Truglio,[1] Giacomo Grasselli,[1,3] Alberto Zanella,[1,3] Stefania Villa,[1] Luca Valenti,[1,3,†] and Daniele Prati[1,†]

[1]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [2]Istituto Europeo di Oncologia IRCCS, Milan, Italy; and [3]Università degli Studi di Milano, Milan, Italy

The direct antiglobulin test (DAT) detects immunoglobulin or complement bound in vivo to red blood cells (RBCs) and is widely used to diagnose immune-mediated hemolytic anemias. Positive DAT results, with or without clinically evident anemia, have been reported in a subset of patients with various viral infections.[1] Very recently, a few cases with simultaneous onset of SARS-CoV-2 infection and autoimmune hemolytic anemia (AIHA) have been described.[2,3]

During the first weeks of the coronavirus disease 2019 (COVID-19) outbreak, we noticed an increasing frequency of DAT positivity at the Blood Center of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy). Therefore, we studied samples from 113 consecutive patients with confirmed COVID-19 that were sent to our laboratory for pretransfusion testing and/or AB0 and Rh typing during a single week (6-13 April 2020). All patients were hospitalized and receiving treatment with multiple drugs (including hydroxychloroquine, heparin, corticosteroids, anti–interleukin-1 biologicals, antivirals, antibiotics, vasopressors, and invasive or noninvasive ventilation). None of them received COVID-19 convalescent plasma. Red cell investigations were performed at the Immunohematology Reference Laboratory of the Department of Transfusion Medicine and Hematology of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, which is certified by the American Association of Blood Banks. The study was approved by the Institutional Review Board of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and conducted in accordance with the Declaration of Helsinki.

Red cell antibodies were determined by direct antiglobulin test and indirect antiglobulin test (IAT), using column agglutination technology (ORTHO BioVue system; Ortho Clinical Diagnostics, Raritan, NJ) and polyspecific antiserum (immunoglobulin G [IgG] plus C3d) and specific antiserum (IgG). DAT reactivity was confirmed, and antibody specificity was determined by microcolumn (IgG, IgA, IgM, C3c, C3d; DC-Screening I; Bio-Rad, Cressier, Switzerland). Reagent cells from patients and healthy donors were prepared from EDTA samples by washing the red cell fraction 3 times with saline. Specifically, we prepared 1 panel of 5DAT-negative patients with COVID-19 and 1 panel of 5 blood donors. A commercial panel of RBCs (SURGISCREEN; Ortho Clinical Diagnostics) was also used. Rapid acid elution (Gamma ELU-KIT II; Immucor Inc., Norcross, GA) was used for the recovery of antibodies bound to red cells, and the eluates were tested by IAT.

Fifteen consecutive samples were also tested by flow cytometry DAT. Briefly, packed RBCs were diluted with phosphate-buffered saline to reach a 1:80 suspension, and 10 μL were incubated with 50 μL of fluorescein isothiocyanate–conjugated $F(ab')_2$ goat anti-human IgG (Invitrogen, Carlsbad, CA) for 45 minutes at room temperature. After washing, samples were acquired using a BD FACSLyric flow cytometer, and the data were analyzed using FACSuite software (both from BD Biosciences, San Jose, CA). Each assay included a positive control (CHECKCELL; Immucor Gamma, Houston, TX) to validate the testing procedure. Results were expressed as median fluorescence intensity.

SARS-CoV-2 RNA viremia was determined in a subset of consecutive patient samples (N = 10) at the Laboratory of Hematology/Oncology, Istituto Europeo di Oncologia. RNA was extracted from 1200 μL of plasma and 600 μL of packed cellular fraction using a QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) and tested by SARS-CoV-2 RNA Droplet Digital PCR System (QX200; Bio-Rad) with Centers for Disease Control and Prevention primers and probes (2019-nCoV Kit;

Table 1. Clinical and laboratory data for COVID-19 patients

	All patients (N = 113)	DAT-positive patients (n = 52)	DAT-negative patients (n = 61)	P*
Age, mean ± SD, y	63.5 ± 17.1	65.2 ± 16.2	62.1 ± 17.9	.17
Males, n (%)	60 (53.1)	29 (55.8)	31 (50.8)	.60
Length of hospital stay, d	15.5 (10-26)	19 (10.2-30)	14 (9-22.2)	.06
Hemoglobin, g/L	11.2 (8.6-12.7)	9.8 (7.9-11.7)	12.2 (9.4-13.1)	**.001**
Red cell distribution width, %	14 (13.2-15.2)	14.5 (13.5-15.9)	13.6 (12.7-14.4)	**.001**
Platelet count, $\times 10^9$/L	260 (183-335)	308 (207-395)	237 (162-303)	.06
Mean platelet volume, fL	10.7 (10.1-11.5)	10.6 (9.9-11.5)	10.9 (10.1-11.5)	.82
LDH, U/L	229 (199.5-343.5)	247 (203.5-351)	227.5 (184.5-316)	.39
Total bilirubin, mg/dL	0.51 (0.32-0.71)	0.52 (0.36-0.80)	0.48 (0.33-0.66)	.63
Serum ferritin, μg/L	782 (452-1227)	922 (526-1320.5)	702 (359.5-1142.7)	.24
D-dimer, μg/L	1269 (711-2427)	1578 (803.5-3338.5)	1109 (635-2074.5)	.11
C-reactive protein, mg/dL	4.13 (1.42-10.32)	6.27 (1.81-10.79)	2.83 (1.12-9.11)	.12
Patients receiving ≥1 transfusion, n (%)	44 (39)	27 (51.9)	17 (27.9)	**.009**
Units of RBCs transfused per patient, n	0 (0-2)	1 (0-2)	0 (0-1)	**.005**
Patients on endotracheal intubation, n (%)	18 (16)	11 (22)	7 (11.5)	.13
Mortality at 30 d, n (%)†	14 (12.4)	8 (15.4)	6 (9.8)	.37

Unless otherwise noted, data are median (interquartile range). Data for transfusion requirements were calculated during follow-up after DAT determination. Reference laboratory values were as follows: red cell distribution width, 11.5% to 14.5%; mean platelet volume, 9.5 to 13.1 fL; LDH, 135 to 214 U/L; total bilirubin, 0.12 to 1.1 mg/dL; serum ferritin, 15 to 150 μg/L; D-dimer <500 μg/L; and C-reactive protein <0.5 mg/dL. Numbers in bold type indicate statistical significance.

*A χ^2 test was used for discrete variables, and a Student *t* test or a Wilcoxon-Mann-Whitney test was used for continuous variables. Data were compared by generalized linear model (unadjusted).

†Mortality at 30 days was calculated from the date of DAT determination. Other data refer to the day of DAT determination.

Integrated DNA Technologies, Coralville, IA), with a lower limit of detection of 0.109 copies per microliter.[4]

A positive DAT was found in 52 of 113 (26%) COVID-19 patients using a microcolumn screening assay. This prevalence of DAT reactivity was substantially higher than that observed at the Blood Center of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico among non–COVID-19 transfusion candidates (<10%; S.V., N.R., and D.P., unpublished data) and that reported by other investigators in hospitalized patients with acute illness (1-15%).[1] Forty-six patients (88%) tested positive for IgG only, 4 (8%) tested positive for IgG plus C3d, and 2 (4%) tested positive for C3d only. Positive cases were confirmed by at least another microcolumn method and, in a subgroup, by flow cytometry, ruling out the hypothesis of a technical artifact. These results were obtained before patients received blood transfusions.

In contrast to what is commonly observed in cases of AIHA, no patient sera or eluates (ie, the solution of antibodies recovered from the RBC surface of DAT-positive patients) reacted by IAT against a commercial panel of reagent RBCs or with healthy donors' RBCs. However, all eluates containing IgG tested positive with a panel of RBCs prepared from DAT-negative COVID-19 patients. These data suggest that the mechanism underlying DAT reactivity involves modifications of the erythrocyte surface during the course of the disease. A possible interpretation is that hyperinflammation in COVID-19 enhances the deposition of complement C3 and the binding of IgG autoantibodies to RBC membranes, which promotes the clearance of damaged RBCs by macrophages.[5] In a subset of 78 patients, we could not demonstrate any association between DAT reactivity and the medications administered during hospitalization. Nevertheless, data on medications were incomplete and not prospectively collected; therefore, we cannot rule out the possible contribution of drug-induced mechanisms. We also considered the possibility that DAT reactivity occurs as a consequence of antibodies directed to viral proteins bound to the RBC membrane. However, this seems unlikely, because in all patients that we tested, SARS-CoV-2 RNA was not detectable in plasma or RBCs using a highly sensitive droplet digital polymerase chain reaction protocol.

We next analyzed the possible relationship between positive DAT results and patients' clinical and laboratory characteristics retrieved from the electronic clinical record (Table 1). Our data indicate that the presence of membrane-bound immunoglobulins was related to the severity of anemia, because DAT-positive patients had lower hemoglobin concentrations, greater anisocytosis, and needed

more transfusions than DAT-negative patients ($P < .01$ for all). However, total bilirubin and lactate dehydrogenase (LDH) concentrations were not different between DAT-positive and DAT-negative cases, and the association with other hemolysis indicators could not be assessed, because reticulocyte count and haptoglobin are not routinely monitored in patients with COVID-19. Therefore, it is possible that anemia was not caused by extravascular hemolysis and, rather, can be interpreted as a marker of advanced disease. Although the numbers of patients requiring endotracheal intubation and mortality at 30 days were not significantly different in the 2 groups, definitive conclusions on disease outcome would require a prospective study design and perhaps a greater number of observations. It remains to be investigated whether RBC membrane injury could promote the thrombotic complications frequently observed in COVID-19 patients.[6]

Finally, the high rate of DAT reactivity can have an impact on pretransfusion testing, because membrane-bound autoantibodies may mask the concomitant presence of RBC alloantibodies in patients who have been recently transfused, and it may complicate or delay the selection of phenotypically matched blood units.[7]

In conclusion, anti-RBC antibodies were detectable in almost half of the patients with COVID-19 referred to our blood center. The serologic features of DAT reactivity in COVID-19 patients are different from those generally observed in AIHA. Nevertheless, this condition was associated with an increasing frequency of anemia and greater transfusion requirements. Our data add more evidence to the importance of immune-mediated mechanisms in the pathogenesis of COVID-19.[8-10]

Acknowledgments

This work was supported in part by grants from Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Ricerca Corrente 2020 and COVID-19 biobank) (L.V. and D.P.) and by grants from Fondazione AIRC per la Ricerca sul Cancro and the Italian Ministry of Health (F.B.).

Authorship

Contribution: A. Berzuini, C.B., L.V., and D.P. wrote the manuscript; C.P., G. Gregato, A.C, L.P., N.R., and F.T. conducted laboratory experiments; and all authors contributed to the acquisition or analysis of data, critically revised the manuscript, approved the final version for publication, and agreed to be accountable for the results published.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: A.C., 0000-0002-4500-6540; M.M., 0000-0002-1875-5335; L.P., 0000-0001-8100-4262; G.Grasselli, 0000-0002-1735-1400; A.Z., 0000-0002-2967-2527; L.V., 0000-0001-8909-0345; D.P., 0000-0002-2281-7498.

Correspondence: Daniele Prati, Department of Transfusion Medicine and Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza, 35, 20122 Milano, Italy; e-mail: daniele.prati@policlinico.mi.it.

Footnotes

*A. Berzuini and C.B. contributed equally to this work.

†L.V. and D.P. contributed equally to this work.

Data sharing requests should be sent to Daniele Prati (daniele.prati@policlinico.mi.it).

There is a *Blood* Commentary on this article in this issue.

REFERENCES

1. Parker V, Tormey CA. The direct antiglobulin test: indications, interpretation, and pitfalls. *Arch Pathol Lab Med*. 2017;141(2):305-310.
2. Lopez C, Kim J, Pandey A, Huang T, DeLoughery TG. Simultaneous onset of COVID-19 and autoimmune haemolytic anaemia. *Br J Haematol*. 2020; 190:31-32.
3. Lazarian G, Quinquenel A, Bellal M, et al. Autoimmune haemolytic anaemia associated with COVID-19 infection. *Br J Haematol*. 2020;190:29-31.
4. Suo T, Liu X, Feng J, et al. ddPCR: a more sensitive and accurate tool for SARS-CoV-2 detection in low viral load specimens. *Emerg Microbes Infect*. 2020;9(1):1259-1268.
5. Straat M, van Bruggen R, de Korte D, Juffermans NP. Red blood cell clearance in inflammation. *Transfus Med Hemother*. 2012;39(5):353-361.
6. Bikdeli B, Madhavan MV, Jimenez D, et al. COVID-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up. *J Am Coll Cardiol*. 2020;75(23):2950-2973.
7. Branch DR, Petz LD. Detecting alloantibodies in patients with autoantibodies. *Transfusion*. 1999;39(1):6-10.
8. Zhang Y, Xiao M, Zhang S, et al. Coagulopathy and antiphospholipid antibodies in patients with Covid-19. *N Engl J Med*. 2020;382(17):e38.
9. Zulfiqar AA, Lorenzo-Villalba N, Hassler P, Andrès E. Immune thrombocytopenic purpura in a patient with Covid-19. *N Engl J Med*. 2020;382(18):e43.
10. Toscano G, Palmerini F, Ravaglia S, et al. Guillain-Barré syndrome associated with SARS-CoV-2. *N Engl J Med*. 2020;382:2574-2576.

DOI 10.1182/blood.2020006695