Signal transduction pathway involved in platelet activation in immune thrombotic thrombocytopenia after COVID-19 vaccination

The severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection is currently advancing and is exponentially developing the COVID-19 pandemic, especially in the USA, Europe, South America, Russia, and India, recording more than 3,000,000 deaths. The outbreak of diseases has significantly impacted the lives of millions of people and within a year, several vaccines have been developed to control the pandemic. The European Medicines Agency (EMA), on the basis of randomized, blinded, controlled trials, validated four different vaccines, among them ChAdOx1 nCoV-19 (AstraZeneca), a recombinant chimpanzee adenoviral vector encoding the spike glycoprotein of SARS-CoV-2.

The condition of great world emergency has imposed very tight times for the experimentation and controlled trials of these vaccines. Since many people are vaccinated and follow-up is extensive, it might even be possible that new vaccine-related adverse events will arise.

Recently, four studies published by New England Journal of Medicine have described a syndrome characterized by thrombosis and thrombocytopenia that came up 5 to 24 days after initial vaccination with ChAdOx1 nCoV-19 (AstraZeneca). Patients described in the studies were healthy or in medically-stable condition without previous history of thromboses. Notably, a high percentage of the patients had thrombosis at unusual sites in particular at cerebral venous sinus with a median platelet count at diagnosis of approximately 20,000 to 30,000 per cubic millimeter.

In addition to the signs and symptoms of the syndrome and the post-vaccination concomitance, the interesting fact that correlates the individual cases with each other is the high level of antibodies to platelet factor 4 (PF4)-polyanion complexes. These are platelet-activating antibodies which clinically mimic autoimmune heparin-induced thrombocytopenia (HIT). Indeed, in some heparin-treated patients, the drug combines with a protein produced by platelets, PF4, to form a complex. The binding of the anti-heparin/PF4 antibody to the heparin-PF4 complex activates the platelets, leading to their aggregation and thrombocytopenia.

So far there are very few hypotheses on the pathophysiological mechanisms of post-vaccination PF4-polyanion antibodies.

Some studies described the occurrence of antiphospholipid antibodies (aPL) in some patients vaccinated with ChAdOx1 nCoV-19, suggesting that a wider spectrum of antibodies may play a role in the pathogenesis of vaccine-induced immune thrombotic thrombocytopenia (VITT). Some of the authors have described two cases of malignant middle cerebral artery (MCA) infarction with a concomitant thrombocytopenia within 10 days after vaccination with ChAdOx1 nCoV-19.

Thus, in the present study we analyze in these two patients antibody specificity and the signaling transduction pathway involved in platelet activation in detail.

At the laboratory of the Autoimmunity Unit of the Umberto I Polyclinic of Rome (UTN Unit), we processed sera from two patients (female, age 57 and 55 years, respectively) with VITT, admitted to the UTN Unit, and a serum from a healthy donor vaccinated with ChAdOx1 nCoV-19 (non-hospitalized female subject, age 52 years). We obtained immunoglobulin G (IgG) fractions by using protein G-Sepharose columns. Patient 1 revealed right middle cerebral artery occlusion and severe thrombocytopenia (44,000/mm³); patient 2 showed occlusion of the right internal carotid artery terminus and of the left middle cerebral artery, with mild thrombocytopenia (133,000/mm³). Both patients had extensive lung and portal vein thrombosis.

The study was conducted in compliance with the Declaration of Helsinki and the local Ethical Committee approved this study (clinicaltrials.gov. Identifier: NCT04844632). For blood samples, healthy donors and relatives of both patients gave written informed consent.

IgG fractions were used to stimulate platelets isolated from healthy donors (females, age range, 36-40 years), who gave written informed consent. Blood samples, in the presence of sodium citrate as anticoagulant, were centrifuged at 150 g for 15 minutes (min) at 20°C to obtain platelet-rich plasma (PRP). Two-thirds of the PRP were drawn, without disturbing the buffy coat layer, in order to prevent contamination. PRP was then mixed with ACD to avoid platelet activation, and centrifuged at 900 g for 10 min at 20°C. Platelet-poor plasma (PPP) was discarded and platelet pellets were resuspended with calcium-free Tyrode’s buffer, containing 10% (v/v) ACD and washed as above. Then, platelets were resuspended in calcium-free Tyrode’s buffer with the addition of bovine serum albumin (BSA, 3 mg/mL). The purity of the isolated platelets was verified by staining with a fluorescein isothiocyanate (FITC)-conjugated anti-CD61 monoclonal antibody (mAb) (Beckman Coulter, Hialeah, FL, USA) and analyzed by flow cytometry (CytoFLEX, Beckman Coulter), as shown in the Online Supplementary Figure S1A.

Since the p38 mitogen-activated protein kinase (MAPK) pathway is an important intracellular signaling pathway in platelets which can be activated by various stimuli and may be an integral component of arterial and venous thrombosis, we analyzed by western blot the effect of IgG fractions from these patients on ERK and p38 phosphorylation in platelet lysates.

For this purpose, human platelets, untreated or treated with healthy donor IgG or patient IgG fractions, for 10 min at 37°C, were resuspended in lysis buffer containing 20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF and 1 mM Na3VO4, including protease inhibitors. Then, whole extracts proteins (40 µg/sample) were separated in 10% SDS-PAGE. Proteins were electrothermally transferred to PVDF membranes (Bio-Rad Laboratories, Richmond, CA, USA) and then, after blocking with Tris-buffered saline Tween 20 (TBS-T) 3% BSA, incubated with polyclonal rabbit anti-phospho-ERK1/2 (Cell Signaling, Inc., Danvers, MA, USA), polyclonal rabbit anti-phospho-p38 antibodies (Cell Signaling, Inc.) Antibody reactions were visualized by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma-Aldrich, Milan, Italy), and then by the chemiluminescence reaction, using enhanced chemiluminescence western blot system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Parallel experiments, human platelets were treated with healthy donor IgG or patient IgG fractions for 4 hours at 37°C, pretreated with ERK inhibitor PD98059 (10 µM, 3 min at 37°C). Then, platelet samples were lysed and analyzed in western blot as above using rabbit anti-tissue factor (TF) mAb (ab228968, Abcam, Cambridge, UK). Data obtained are expressed as means ± standard deviation (SD) of at least
Figure 1. Immunoglobulin G fractions from patients induce ERK and p38 phosphorylation with tissue factor expression. Human platelets from healthy donors were treated with immunoglobulin G (IgG) fractions from the 2 patients and from a healthy donor vaccinated with ChAdOx1 nCoV-19 (10 minutes for ERK and p38 activation and 4 hours for tissue factor [TF] analysis). Protein extracts were separated by SDS-PAGE and analyzed by western blot to investigate phosphorylated ERK (A), p38 MAPK (B), and TF (C), using anti-phospho-ERK1/2, anti-phospho-p38 and anti-TF antibodies, respectively. Data are reported as mean ± standard deviation from 3 independent experiments. Statistical analysis indicated: **P<0.001 vs. untreated; §§P<0.001 vs. healthy donor IgG fraction; § P=0.01 vs. healthy donor IgG fraction; °°P<0.001 vs. pretreated with PD98059.
three independent experiments. Statistical analysis was performed by the paired Student’s t-test. Statistical significance was set up at P<0.01.

Results show that the treatment with both IgG fractions from patients with VITT, induced a significant increase of both phospho-ERK (Figure 1A) and phospho-p38 expression (Figure 1B) compared to untreated platelets. In the presence of ERK inhibitor PD98059, TF expression in platelets was significantly decreased. Moreover, pretreatment with ERK inhibitor PD98059 partially prevented TF expression in samples stimulated with patient IgG fractions (Figure 1C), indicating an increased level of TF expression in VITT patients compared to healthy controls.

Surface TF expression was also verified by flow cytometry analysis, which revealed that the increase in TF expression was not only due to platelet activation, but was also caused by the IgG fraction from VITT patients. The treatment with IgG fraction from VITT patients induced a significant increase of TF levels in platelets (Figure 1D), indicating the involvement of the platelet activation pathway(s) leading to platelet activation and TF expression increase with a consequent amplification of the clotting cascade. We confirmed and extended the analysis of aPL, also showing the presence of “unconventional” (“non-criteria”) aPL in serum of our patients.

The pathogenic mechanism may be consequent to ChAdOx1 nCov-19 vaccination remains unclear; however, a possible pathogenic role of the adenoviral viral vector cannot be excluded.

Serological characteristics of the patients

Table 1. Serological characteristics of the patients

<table>
<thead>
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<th>Test</th>
<th>Healthy donor</th>
<th>Patient 1</th>
<th>Patient 2</th>
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<td>aPF4 IgG (GPL/mL)</td>
<td>0</td>
<td>10.4</td>
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<td>aCL IgG (GPL/mL)</td>
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<td>0</td>
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<td>2.9</td>
<td>0</td>
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<td>3.2</td>
<td>0.7</td>
</tr>
<tr>
<td>LA</td>
<td>0.95</td>
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these patients are able to trigger a signal transduction pathway involving ERK and p38 MAPK, which may lead to TF expression increase with a consequent amplification of the clotting cascade. We cannot exclude the possibility that other additional signal transduction pathways may be involved.

However, other autoantibody specificities have been described in sera of patients affected by VITT, including aPF4 and/or LA. This finding may be relevant, since anti-b2GPI/b2GPI complexes were shown to induce signal transduction pathway(s) leading to platelet activation, particularly promoting thrombosis via p38 MAPK and consequent involvement of TF as major initiator of the clotting cascade. We confirmed and extended the analysis of aPL, also showing the presence of “unconventional” (“non-criteria”) aPL in serum of our patients.

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