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SHORT REPORT

Production of anti-PF4 antibodies in antiphospholipid antibody-positive patients is not affected by COVID-19 vaccination

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ABSTRACT

Background Antibodies against cationic platelet chemokine, platelet factor 4 (PF4/CXCL4), have been described in heparin-induced thrombocytopenia (HIT), but also in patients positive for antiphospholipid antibodies (aPL) even in the absence of heparin treatment and HIT-related clinical manifestations. Anti-PF4 antibodies have been recently described also in subjects who developed thrombosis with thrombocytopenia syndrome (TTS) in association with adenoviral vector-based, but not with mRNA-based, COVID-19 vaccines.

Objective To investigate whether COVID-19 vaccination affects the production of anti-PF4 antibodies in aPL-positive patients and in control groups.

Methods Anti-PF4 immunoglobulins were detected in patients' and controls' serum samples by ELISA and their ability to activate normal platelets was assessed by the platelet aggregation test.

Results Anti-PF4 were found in 9 of 126 aPL-positive patients, 4 of 50 patients with COVID-19, 9 of 49 with other infections, and 1 of 50 aPL-negative patients with systemic lupus erythematosus. Clinical manifestations of TTS were not observed in any aPL patient positive for anti-PF4, whose serum failed to cause platelet aggregation. The administration of COVID-19 vaccines did not affect the production of anti-PF4 immunoglobulins or their ability to cause platelet aggregation in 44 aPL-positive patients tested before and after vaccination.

Conclusions Heparin treatment-independent anti-PF4 antibodies can be found in aPL-positive patients and asymptomatic carriers, but their presence, titre as well as in vitro effect on platelet activation are not affected by COVID-19 vaccination.

Key messages

- Antiplatelet factor 4 (anti-PF4) antibodies have been described both in heparin-induced thrombocytopaenia (HIT) and in antiphospholipid antibodies (aPL)-positive patients independently from heparin treatment and HIT-related clinical manifestations.
- Anti-PF4 antibodies have been detected after administration of adenoviral vector-based, but not mRNA-based, COVID-19 vaccines and have been associated with thrombosis with thrombocytopaenia syndrome (TTS).
- Low-titre, heparin-dependent and platelet aggregation test-negative anti-PF4 antibodies have been found in a small proportion of aPL-positive patients, as well as in aPL-negative patients with systemic lupus erythematosus and infectious diseases.
- COVID-19 vaccination neither affects the titre of pre-existing anti-PF4 antibodies in aPL-positive patients nor induces the ability of these antibodies to activate platelets in vitro.
- Vaccines against COVID-19 are seemingly safe and unable to induce clinical and laboratory TTS-associated manifestations in aPL-positive patients.

INTRODUCTION

Patients with heparin-induced thrombocytopenia (HIT) display immunoglobulins reacting with cationic platelet chemokine, platelet factor 4 (PF4/CXCL4). Comparable antibodies were described also in patients positive for antiphospholipid antibodies (aPL) even in the absence of treatment with heparin and HIT-related clinical manifestations.[1–7] Despite the heterogeneity of published data, majority of the studies reported the presence of anti-PF4 antibodies in up to 21% of aPL-positive samples, their heparin-dependent binding as in HIT but at lower titre and with no effect on platelet activation.

Anti-PF4 antibodies have also been recently described in COVID-19 vaccine-associated

thrombosis with thrombocytopenia syndrome (TTS).[8–11] Although the hypothesis of a molecular mimicry between self-autoantigens and SARS-CoV-2 spike (S) protein is still debated, the active immunisation with S protein was suggested to be responsible for the antibody response against PF4 as well.[12 13] Accordingly, the issue of a potential danger of COVID-19 vaccination in aPL-positive patients was raised because of their thrombophilic state and the possible occurrence of anti-PF4 antibodies in some of them. With this in mind, we investigated whether COVID-19 vaccination affects the production of anti-PF4 antibodies in aPL-positive patients and their functional ability to induce in vitro platelet activation.

METHODS

Patients

We investigated 126 aPL-positive samples, including 71 primary antiphospholipid syndrome (PAPS), 37 aPL-positive asymptomatic carriers and 18 antiphospholipid syndrome associated with systemic autoimmune rheumatic disorders (SAPS). The diagnoses were made as previously described[14]; all samples displayed double or triple positivity for the APS laboratory classification criteria,[14] and medium/high titres of anticardiolipin and anti-beta2 glycoprotein I IgG/IgM.

As control groups the following samples were also tested: 50 patients with COVID-19 with moderate disease as previously reported,[15] 49 individuals with non-COVID-19 infections (9 Epstein-Barr virus, 2 hepatitis C virus, 14 rubella virus, 14 cytomegalovirus, 10 syphilis) and 50 aPL-negative patients with systemic lupus erythematosus (SLE).[16]

Nineteen PAPS, 12 aPL-positive asymptomatic carriers and 13 SAPS were tested before and after COVID-19 vaccination. Majority of the patients (38 of 44) were vaccinated with Comirnaty. A handful of patients received other vaccines: of 44 patients, 2 received Spikevax, 3 Vaxzevria and 1 Sputnik. Two additional patients with PAPS were tested before and after full-blown COVID-19 and positivity for SARS-CoV-2 nasopharyngeal swab confirmed by PCR. One hundred and fifty healthcare workers were also enrolled and serum samples were collected before and after vaccination by Comirnaty (100) or Vaxzevria (50).

Samples were collected before the second vaccine injection for both aPL-positive patients and healthcare workers (3 weeks after Comirnaty or Sputnik, 4 weeks after Spikevax, and 12 weeks after Vaxzevria first injection, respectively).

Severe adverse side effects as defined by Polack *et al*[17] or any clinical manifestations potentially correlated with vaccination were also recorded for all the investigated patients or subjects (online supplemental table).

All patients/subjects gave their informed consent.

Anti-PF4 detection

Anti-PF4 IgG/IgA/IgM were assessed by the polyspecific Lifecodes PF4 Enhanced ELISA (Immucor, Solihull, UK). The assay was performed according to the manufacturer's instructions, including negative and positive controls and confirmatory inhibition of the reaction in the presence of high concentrations of heparin (100 U/mL). Anti-PF4 antibodies were detectable in 1.0%–4.3% of normal healthy subjects (NHS), depending on the commercial kit used.[18] Due to variability in results, we used our inhouse cut-off value of 0.80 optical density (OD) units, which was calculated as the mean +3 SD of the results obtained in 189 NHS. Samples with binding values >0.80 OD were retested in the presence of heparin (100 IU/mL) and for their ability to induce platelet aggregation (platelet aggregation test, PAT).

Platelet aggregation test

PAT was performed using washed human platelet (WP) suspensions prepared as described[19] from citrate-dextrose (ACD)-anticoagulated blood from NHS and resuspended with Tyrode's solution without added $CaCl_2$, containing 0.01 U/mL Apyrase from potatoes (Sigma-Aldrich, Taufkirchen, Germany). Heat inactivated (56°C, 30 min) serum was added to WP with buffer or heparin (0.2 and 100 IU/mL) (Veracer; Medic Italia, Milan, Italy). Platelet aggregation was measured in the PAP-8E Platelet Aggregation Profiler (Bio/Data Corporation, Horsham, Pennsylvania, USA) for 30 min.

RESULTS

Prevalence of anti-PF4 immunoglobulins in the study groups

Figure 1 shows the prevalence of anti-PF4 immunoglobulins in the study groups. Values of anti-PF4 immunoglobulins higher than the cut-off value of 0.80 OD were found in 9 of 126 (7%) aPL-positive samples, 1 of 50 (2%) aPL-negative patients with SLE, 4 of 50 (8%) patients with COVID-19, and 4 of 49 (18%) patients suffering from non-COVID-19 infectious diseases. The antibody binding in the presence of excess of heparin (100 IU/mL) was significantly inhibited in all positive samples. Moreover, the titres of anti-PF4 immunoglobulins were lower than those usually described in patients with HIT and no HIT-related clinical manifestations were recorded.[11]

Variations of anti-PF4 immunoglobulin titres before and after COVID-19 vaccination

Figure 2A shows the variations in anti-PF4 antibody reactivity before (T0) and after (T1) COVID-19 vaccination in aPL-positive patients classified as PAPS, SAPS or asymptomatic aPL-positive carriers. No significant changes in antibody titres were found in all the investigated patients. Two additional patients with PAPS were tested before and 1 month after full-blown COVID-19 of moderate severity and did not show any modification in the titres (PAPS1: 0.476 OD and 0.423 OD; PAPS2: 0.443 OD and 0.788 OD, before and after COVID-19, respectively).

Anti-PF4 immunoglobulin titres in healthcare workers before and after COVID-19 vaccination are shown in figure 2B. Anti-PF4 immunoglobulins at low titres were found at baseline in 2 of 100 (2%) Comirnaty and in 1

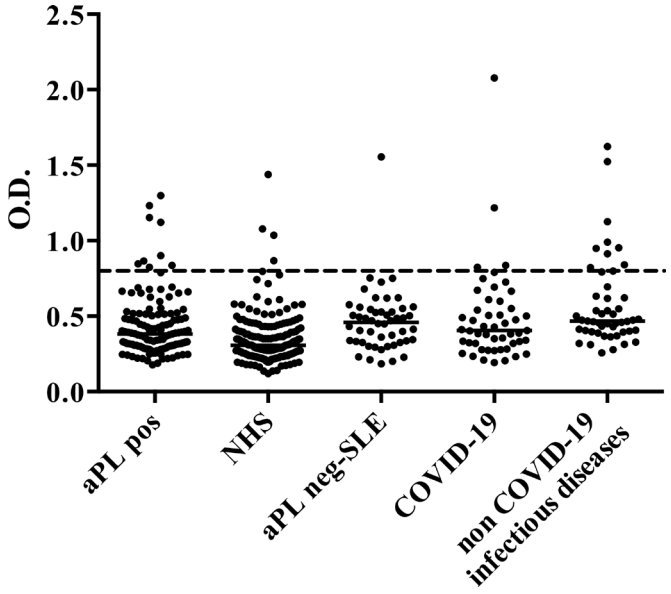

Figure 1 Binding activity of anti-PF4 immunoglobulins in the study groups. Anti-PF4 binding activity of serum samples from aPL-positive patients or carriers (aPL pos), NHS, patients with SLE negative for aPL (aPL neg-SLE), patients with COVID-19 or patients with non-COVID-19 infections. Data are expressed as OD. Dashed line indicates the inhouse cut-off value (0.80 OD). There was no statistically significant difference among the groups. aPL, antiphospholipid antibodies; NHS, normal healthy subjects; OD, optical density; PF4, platelet factor 4; SLE, systemic lupus erythematosus.

of 50 (2%) Vaxzevria vaccinated subjects. An increase in the antibody titre was found in one subject only (from 0.174 OD to 1.682 OD), with no TTS-related symptoms and normal platelet count. No clinical manifestations related to TTS were recorded for all the other subjects included in the study. The sera positive for anti-PF4 immunoglobulins at OD values >0.80 were also tested in the PAT but none of them resulted positive (data not shown). Three healthcare workers received Comirnaty and one Vaxzevria; none was receiving any anticoagulant or antiplatelet drug. Four aPL-positive patients received Comirnaty; one patient was on direct oral anticoagulant, one on low-dose aspirin and two on vitamin K antagonist. The possible interference of the therapy on PAT is unlikely since the pretreatment of the sera inactivates coagulation cascade components that are targets of the oral anticoagulant drugs. Moreover, the potential effect of low-dose aspirin on platelet aggregation was minimised by collecting the serum in the morning, far from the drug taken at lunchtime.

DISCUSSION

Anti-PF4 immunoglobulins at low titre are detectable in a minority of healthy subjects and in different pathological conditions. In particular, our data show a prevalence of anti-PF4 positivity in SLE similar to that of the largest studies published in the literature.[1–7] These autoantibodies have been defined as 'false-positive tests for HIT' because they are not associated with HIT-related clinical manifestations and do not trigger in vitro platelet activation.[1–7] Nevertheless, most of them display an in vitro heparin-dependent binding activity in spite of no treatment with heparin.[1–7] We confirmed and extended this finding showing low-titre, heparin-dependent and PAT-negative anti-PF4 antibodies in a large series of aPL-positive patients, as well as in aPL-negative patients with SLE and infectious diseases.

In agreement with others and in contrast to the data reported by Pauzner *et al*,[1] we found a low prevalence of anti-PF4 immunoglobulins in PAPS, SAPS and SLE.[2–7] The addition of an excess of heparin strongly inhibited antibody binding in the solid-phase assay in all samples, suggesting that the autoantibodies were heparin-dependent. At variance with the antibodies detectable in HIT, anti-PF4 antibodies in aPL-positive patients were at medium/low titre and without any platelet activation effect, even in the presence of low heparin concentration (0.2 IU/mL).

COVID-19 vaccination with adenovirus-based vaccines may trigger TTS associated with the presence of high-titre anti-PF4 antibodies, which may trigger in vitro platelet

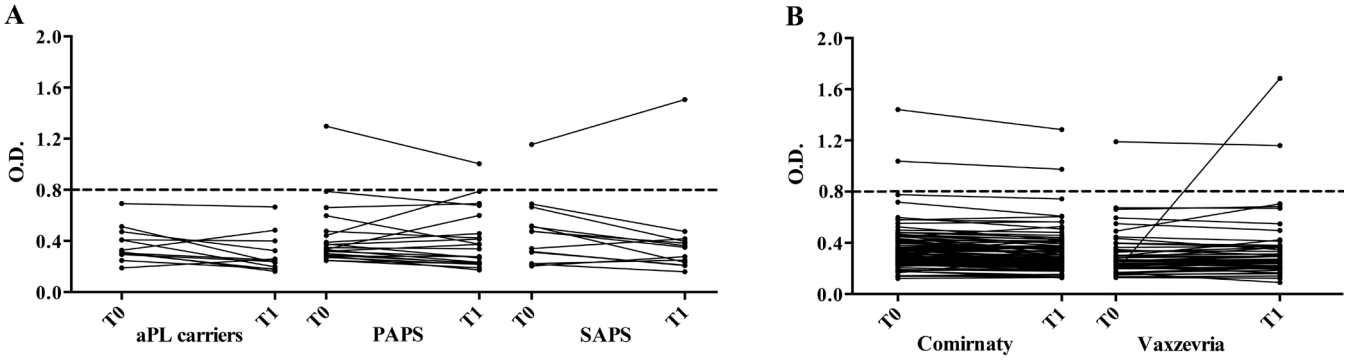

Figure 2 Effect of COVID-19 vaccination on anti-PF4 immunoglobulin titres. (A) Anti-PF4 OD values before (T0) and after (T1) COVID-19 vaccination in asymptomatic aPL-positive carriers and in patients with PAPS and SAPS. (B) Anti-PF4 OD values before (T0) and after (T1) Comirnaty or Vaxzevria vaccines in healthcare workers. Dashed lines indicate the inhouse cut-off value (0.80 OD). aPL, antiphospholipid antibodies; OD, optical density; PAPS, primary antiphospholipid syndrome; PF4, platelet factor 4; SAPS, antiphospholipid syndrome associated with systemic autoimmune rheumatic disorders.

activation even in the absence of low concentrations of heparin.[8–11] Whether or not COVID-19 vaccination may increase the titre of pre-existing anti-PF4 antibodies in aPL-positive patients or induce the ability to activate platelets is an issue with clinical implications due to the prevalence of aPL positivity in a small but significant percentage of the general population.[20] Our data show that vaccination against COVID-19 does not trigger ex novo production of anti-PF4 antibodies nor affect the titre of pre-existing antibodies in a well-characterised aPL-positive series. More importantly, vaccination does not enable these antibodies to cause platelet activation in vitro. Comparable data were found in a group of healthcare workers vaccinated with Comirnaty or Vaxzevria, with the exception of the increase of anti-PF4 immunoglobulins in one subject only, without any clinical or laboratory manifestations of TTS.

In summary, anti-PF4 antibodies can be found in a small proportion of aPL-positive patients but with characteristics different from the antibodies detectable in patients with HIT and TTS. COVID-19 vaccination is apparently safe in aPL-positive patients and does not trigger the production of TTS-associated autoantibodies, although larger series of patients vaccinated with adenoviral vector-based vaccines are needed to definitely support our conclusions.

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