A flow cytometric assay to detect platelet-activating antibodies in VITT after ChAdOx1 nCov-19 vaccination

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Vaccination is crucial in combatting the severe acute respiratory syndrome coronavirus 2 pandemic. The rare complication of thrombocytopenia and thrombotic complications at unusual sites after ChAdOx1 nCov-19 vaccination is caused by platelet-activating antibodies directed against platelet factor 4 (PF4). We present a widely applicable whole-blood standard flow cytometric assay to identify the pathogenic antibodies associated with vaccine-induced immune-mediated thrombotic thrombocytopenia (VITT) after ChAdOx1 nCov-19 vaccination. This assay will enable rapid diagnosis by many laboratories. This trial was registered at www.clinicaltrials.gov as #NCT04370119.

Introduction

Coronavirus disease 2019 (COVID-19) is caused by the single-stranded RNA virus severe acute respiratory syndrome coronavirus2 (SARS-CoV-2). Currently, 4 different vaccines to prevent symptomatic COVID-19 have been approved by the European Medicines Agency.1

Recently, several cases of thrombosis combined with moderate to severe thrombocytopenia were observed in patients vaccinated with the ChAdOx1 nCov-19 (AstraZeneca) vaccine2-5 and in patients vaccinated with the Johnson & Johnson vaccine.6,7 We have identified immunoglobulin G antibodies directed against platelet factor 4 (PF4)-activating platelets via FcyRlla to be the likely cause of vaccine-induced immune-mediated thrombotic thrombocytopenia (VITT).3 VITT shows striking similarities with autoimmune heparin-induced thrombocytopenia (HIT).8,9 In contrast to HIT, platelet activation in VITT occurs in the presence of PF4 rather than low heparin concentrations. The antibodies associated with recent ChAdOx1 nCov-19 vaccination can be detected by some PF4/heparin enzyme immunoassays (EIAs),10 which are widely available to diagnose HIT. However, these assays may not be specific for VITT-related antibodies. Not all antibodies binding to PF4/heparin complexes by EIAs are functionally active and others may be typical HIT antibodies. We modified the functional heparin-induced platelet activation test,11,12 a washed platelet assay, to detect vaccine-related antibodies and to differentiate them from HIT antibodies. We have named the modified assay the PF4-induced flow cytometry-based platelet activation (PIPA) test (for details, see supplemental Methods, available on the Blood Web site). As washed platelet assays are restricted to specialized laboratories, we developed a flow cytometric assay using whole blood to detect PF4-dependent platelet-activating antibodies in ChAdOx1 nCov-19–vaccinated patients. By analogy to the washed platelet test, we refer to the modified assay as the PF4-induced flow cytometry-based platelet activation (PIFPA) test.

Study design

For the PIFPA test, citrated whole blood was obtained from healthy donors. Whole blood was supplied with 54 U/mL (final) hirudin (Canyon Pharmaceuticals; if hirudin is not available, then other thrombin inhibitors like D-Phe-Pro-Arg-chloromethylketone [PPACK] can be used), and incubated with 0, 5, or 20 µg/mL PF4 (final) for 20 minutes at 37°C. Afterward, heat-inactivated serum (56°C, 30 minutes) from the following donors was added in a whole-blood-to-serum ratio of 2:1 and incubated for 20 minutes at 37°C: patients with VITT, asymptomatic vaccinated donors being positive or negative in the anti-PF4/heparin EIA, unvaccinated donors, or patients with HIT. Samples were stained using CD61-phycoerythrin (PE) (clone SZ21; Beckman Coulter) and CD62P-PE-Cy5 (Becton Dickinson) antibodies for 10 minutes (room temperature) in the dark. Stained samples were fixed with 2% paraformaldehyde (Morphisto) for 20 minutes, washed in phosphate-buffered saline (pH 7.4; Pan Biotech), centrifuged (650g, 7 minutes, room temperature), and resuspended in...
1× fluorescence-activated cell sorter lysing solution (Becton Dickinson). Samples were measured in a Cytomics FC500 flow cytometer (Beckman Coulter). Platelets were positively gated using CD61-PE. Activation was determined by granule release measured using CD62P-PE-Cy5 and given as mean fluorescence intensity (MFI) of the CD62P⁺ gated events multiplied by the percentage of gated platelets. Platelet activation for each serum was given as median activation from the 4 whole-blood samples. Further details of the test method are given in supplemental Methods.

The use of whole blood and platelets from healthy donors and serum from patients was approved by the Ethics Board at the University Medicine Greifswald and was conducted in accordance with the Declaration of Helsinki.

**Results and discussion**

We tested sera from individuals who developed VITT with thrombosis and thrombocytopenia developing 5 to 16 days after vaccination with ChAdOx1 nCoV-19 as well as postvaccination serum samples from University Hospital Greifswald health care workers vaccinated with ChAdOx1 nCoV-19 (Screening for COVID-19 and Monitoring of Serological Responses to SARS-CoV-2 in Health care Workers [SeCo] study; approved by the Greifswald Ethics Committee, no. BB068/20). Sera were pretested in PF4/heparin EIA and the functional PIPA test. The flow cytometry-based PIFPA test was performed with 16 VITT samples (14 sera, 2 citrated plasma) and all available EIA⁺/PIPA⁻ sera, as well as 10 representative EIA⁻/PIPA⁺ sera and 4 sera of patients with HIT who had a positive EIA and positive HIPA result. Cutoff was determined with sera of 13 unvaccinated healthy controls incubated with 5 µg/mL PF4 as the mean plus 2 standard deviations (SD). With the addition of 5 µg/mL PF4, sera from patients with VITT can be readily discriminated from vaccinated donors with no functionally relevant antibodies (P < .0003). Statistical significance was calculated by the unpaired Student t test. *P < .05; **P < .01; ***P < .001. HIPA, heparin-induced platelet aggregation; UFH, unfractionated heparin.

![Figure 1. Platelet activation in the PIFPA test.](http://ashpublications.org/blood/article-pdf/137/26/3656/1812039/bloodbld2021012064.pdf)
The PIFPA test shows very comparable results to the PIPA test (Table 1) and can be applied to confirm or rule out platelet-activating PF4-dependent antibodies, which occur 5 to 16 days after ChAdOx1 nCov-19 vaccination based on available data, without the need for washed platelets. The exact mechanism whereby vaccination with ChAdOx1 nCov-19 is associated with these platelet-activating anti-PF4 antibodies is still unknown. The disorder appears analogous to autoimmune HIT, where antibodies are capable of inducing strong platelet activation despite the absence of heparin, in contrast to typical HIT sera, which require the addition of heparin. Although the PIFPA seems to be specific, we cannot rule out that patients with less severe complications (e.g., isolated thrombocytopenia, which have not been identified up to now) may have more weakly reacting antibodies induced by ChAdOx1 nCov-19 for which an additional cofactor might be required (by analogy to HIT, akin to heparin) to produce platelet activation by patient serum. The scientific community should undertake all efforts to screen for such a cofactor (or cofactors). In addition, laboratory tests for antibodies causing HIT using washed platelets are always more sensitive and probably even more specific than assays using whole blood.13 We therefore recommend that patients with the typical clinical presentation of VITT, but testing negative in the PIFPA, should be further assessed in the PIPA until more information is available.

In summary, we present an assay to detect platelet-activating antibodies that seem highly specific for VITT after vaccination with ChAdOx1 nCov-19. The pathogenesis of this severe adverse effect, which threatens the vaccination program in the SARS-CoV-2 pandemic, may be further clarified by rapidly identifying the real incidence of VITT caused by platelet-activating antibodies.

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### Authorship

**Contribution:** S.H., M.W., C.Z., and J.W. performed the experiments, analyzed the data, and wrote the manuscript; C.Z., L.S., and T.T. coordinated patient samples and the study protocol; L.U., N.-O.H., and K.B. coordinated the Seco study and provided the samples; K.A. discussed the results and reviewed the manuscript; and A.G. developed the PIPA and the PIFPA concept, supervised the study and experiments, and wrote the manuscript.

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