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Proposed mechanism for rare thrombotic events after use of some Covid-19 vaccines

Peter A. Feldman

Research Unit, Queen's House Consulting, HealthAid House, Marlborough Road, Harrow, Middlesex HA1 1UD, UK

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ABSTRACT

Administration of AstraZeneca/Oxford and Johnson & Johnson/Janssen Covid-19 vaccines which use an adenovirus vector for DNA delivery has been associated with very rare thromboembolic complications coupled with an immune response to platelet factor 4 protein. The cause of this has not yet been identified. It is known that binding of coagulation factor proteins to the surface of some adenoviruses can protect their function. Here I propose that the thromboembolic events are caused by impairment of coagulation factor X binding to the virus capsid. The unprotected capsid then stimulates an immune response leading to platelet activation, increased thrombogenicity and formation of an antibody complex with platelet factor 4. Impaired binding of factor X may be due to an undiagnosed mutation in affected individuals. Options to test this mechanism experimentally and potential remedial actions to resolve the hazard are described. This mechanism offers a remedial route to address concerns about the safety of these vaccines, which are otherwise well-positioned to deliver global Covid-19 immunity across diverse healthcare economies.

Introduction

There are continuing investigations to explain the very rare (less than 0.01%) adverse events associated with thromboembolic complications after administration of the Covid-19 vaccines from AstraZeneca (Vaxzevria injection, Covid-19 Vaccine ChAdOx1-S [recombinant]) [1] and Johnson and Johnson/Janssen (COVID-19 Vaccine Janssen suspension for injection, Covid-19 Vaccine Ad26.COV2-S [recombinant]) [2], but the cause remains unknown. The resulting cautionary guidance from medicines regulators has a negative impact on global adoption of these vaccines, which are otherwise well-suited for use in diverse environmental conditions and health economies. Analysis of blood samples from some of the affected individuals has identified low platelet count and antibodies against platelet factor 4 (PF4), analogous to the pro-thrombotic condition of heparin-induced thrombocytopenia (HIT) [1,3] which is characterised by the formation of antibodies against a complex of heparin and PF4. The similar, but heparin-independent, immune response to an as-yet unidentified characteristic of the vaccine has been described as vaccine-induced immune thrombotic thrombocytopenia (VITT) [3,4]. Possible explanations for the underlying physiology have been reviewed, evaluating their limitations and proposing the experiments needed to test each hypothesis [5]. However, these have not identified a root cause which explains both the particular association of

VITT with adenovirus-vectored vaccines and the very low frequency of these events even within that cohort of vaccinated individuals. Initially, the anecdotal incidence of these adverse events was reported to be between 1 in 500,000 and less than 1 in 1 million (or 0.0001%). However, more recent stratified *meta*-analysis estimates the risk for recipients of Covid-19 Vaccine ChAdOx1-S [recombinant] to be 1 in 1 million for individuals aged 65 years and above, but 1 in 20,000 to 60,000 for individuals below 55 years of age [6]. The incidences are similar to those of inherited blood coagulation factor deficiencies [7], which has prompted consideration of a novel causative mechanism for such very rare adverse events.

Background elements of the hypothesis

Vaccine characteristics

The two vaccines so far associated with these adverse events use different modified adenovirus vectors to deliver DNA encoding for SARS CoV-2 spike protein [8,9]. It is not known whether similar adverse events have occurred following use of two other adenovirus vector DNA vaccines for Covid-19 (Convidecia from CanSino Biologics and Sputnik V/Cam-COVID-Vac from Gamaleya National Centre of Epidemiology and Microbiology). These four vaccines are differentiated from other

E-mail address: paf_research@queenshouseconsulting.co.uk.

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nucleic acid Covid-19 vaccines which utilise mRNA in non-virus vectors. The Astra Zeneca/Oxford vaccine vector ChAdOx1 is a modified chimpanzee adenovirus Y25 which is associated phylogenically with human adenovirus group E and is generally related to human adenovirus serological class 4, although the hexon capsid protein DNA is distinct from Group E human adenoviruses [10]. In contrast, the Johnson & Johnson/Janssen vaccine is derived from human adenovirus 26 which is associated phylogenically with human adenovirus group D [11,12].

Factor X binding to adenovirus

Coagulation factor X protects some adenoviruses (e.g. sub-group C Adenovirus serotype 5) from reaction with immunoglobulin (IgM) and complement. This protective mechanism is independent of the coagulation factor-mediated infection of hepatocyte by adenovirus [13,14,15,16,17]. Protection is achieved by calcium-dependent high-affinity binding of factor X to adenovirus [18]. Each virion can bind approximately 205 factor X molecules via the 240 homotrimeric structures within the adenovirus hexon capsid protein, requiring both specific highly-variable regions on the hexon protein and gamma-carboxylated glutamic acid (Gla) residues located on the factor X molecule [13]. This suggests that factor X binds to negatively-charged amino acids on the hexon protein via a calcium bridge, but does not exclude an additional requirement for other structural properties such as heparin binding sites [16]. The protective effect is achieved by binding factor X zymogen (factor X), without a requirement for activated factor X activity [17].

Although some naturally-occurring adenoviruses (and particularly those in group D) did not show protective binding of factor X using the technique of surface plasmon resonance [13], the factor X binding properties have not been reported specifically for either of the ChAdOx1 or Ad26 Covid-19 vaccine modified adenovirus vectors.

Hypothesis

This hypothesis proposes that the rare thrombotic adverse events after administration of adenovirus-vectored Covid-19 vaccines are due to impairment or absence of protective factor X binding to the adenovirus hexon protein of the ChAdOx1 and Ad26 capsid.

Mechanism for thrombotic consequences of factor X not binding to adenovirus

Without protective factor X binding, some characteristics of the adenovirus capsid hexon protein will be affected by greater exposure to the host environment. In addition to the hexon protein amino acids which naturally carry a negative-charge at neutral pH (aspartic acid and glutamic acid), sulfation of other amino acids may increase the overall negative charge on the virion surface. It has been suggested that up to 1% of all tyrosine residues in proteins undergo secondary sulfation [19], though this may be an overestimate; given the presence of 56 tyrosine residues on the hexon monomer protein [20], there could be up to 240 negatively-charged sulfate groups on the virus capsid (which contains 240 hexon trimers). An adenovirus capsid surface which is not protected by multiple factor X molecules will therefore resemble other polyanionic (and possibly polysulfated) species, promoting interactions which are consistent with the development of VITT. Two of these potential mechanisms are discussed (and depicted schematically in Fig. 1), though others may be conceived [5].

One model (Fig. 1 panel B) promotes binding of IgM to the exposed charged capsid surface along with activation of complement which causes platelet activation, with consequential release of PF4, micro-particles and polyphosphates. The polyphosphates then form an immunogenic complex with PF4 as previously described [21] which gives rise to VITT. In an alternative model (Fig. 1 panel C), the polyanionic (and possibly poly-sulfated) virus capsid surface mimics

heparin, binding endogenous free PF4 to form an immunogenic complex which then stimulates the generation of antibodies. This immune response promotes further platelet activation, causing VITT.

Cause of factor X not binding to adenovirus

Assuming that the modified virus vectors used in Covid-19 DNA vaccines are protected by factor X binding, the models predict that adverse reactions will not occur when there is competent factor X present. This may be achieved during the vaccine manufacturing process, but would require a source of factor X. This cannot be assumed, because factor X will be activated and depleted during the production of originating serum which is an optional component of cell culture medium (but may not even be present in the medium used to manufacture these vaccines). Furthermore, protective binding of factor X to adenovirus is diminished by activation [17] or inhibition [13]. For these reasons, it is unlikely that the virus vector will be manufactured with protective factor X bound to the capsid surface. It follows that any virus protection would instead be derived from binding to endogenous factor X provided by the vaccinated individual. This requires vaccine recipients to have competent factor X, which describes most of the population; after administration, the virus hexon protein will bind endogenous factor X thereby protecting the individual from prothrombotic sequelae and VITT.

However, if an individual lacks competent factor X, protection will not occur and the risk of thrombosis will be greater. Factor X deficiency is a very rare condition, caused by a mutation of the F10 gene on chromosome 13. The incidence of this condition is estimated to be between 1 in 500,000 and 1 in 2 million [7]. This is the same order of magnitude as the estimated incidence of thrombotic events following Covid-19 vaccine administration to individuals aged 65 years and above, but less than the incidence in younger age groups [6]. Clinical factor X deficiency is characterised mostly by missense mutations [7] while a total gene deletion is considered incompatible with life [22]. Known factor X genotype mutations are associated predominantly with clinical bleeding symptoms [23,24], so it is unlikely that an affected individual would reach maturity without diagnosis of a bleeding diathesis. Furthermore, such haemostatically-compromised individuals should be less prone to VITT adverse reactions than the general population. However, a gene mutation which does not affect coagulant activity but does affect adenovirus binding would be undiagnosed in the absence of a clinical prompt for genotyping.

Although different genes may exhibit different mutation rates, the naturally-occurring mutation rate (albeit in the Y-chromosome) has been estimated as 3×10^{-8} mutations/nucleotide/generation [25]. For factor X, containing 488 amino acids (1464 nucleotides), the resulting mutation frequency would be 1 in 23,000, which is 1–2 orders of magnitude greater than the reported incidence of factor X deficiency [7] but of the same order as the estimated incidence of VITT [6]. This suggests potential for more factor X gene mutations than have been identified by clinical diagnosis of impaired haemostasis, though the frequency (less than 1%) would not classify them as genetic polymorphisms. While a proportion of these mutations may have limited clinical significance, it is proposed that some could compromise binding to adenovirus without impairing coagulation function.

Discussion

Evaluation of the hypothesis

A recent meta-analysis estimated the incidence of VITT in the over-65 age group at approximately 1 in 1 million [6] which is the same order of magnitude as the incidence of factor X mutations which cause bleeding diatheses. However, there are acknowledged limitations to this estimation, and greater confidence in the higher incidence value of 1 in 20,000 to 60,000 estimated for the under-55 age group. This remains a very rare

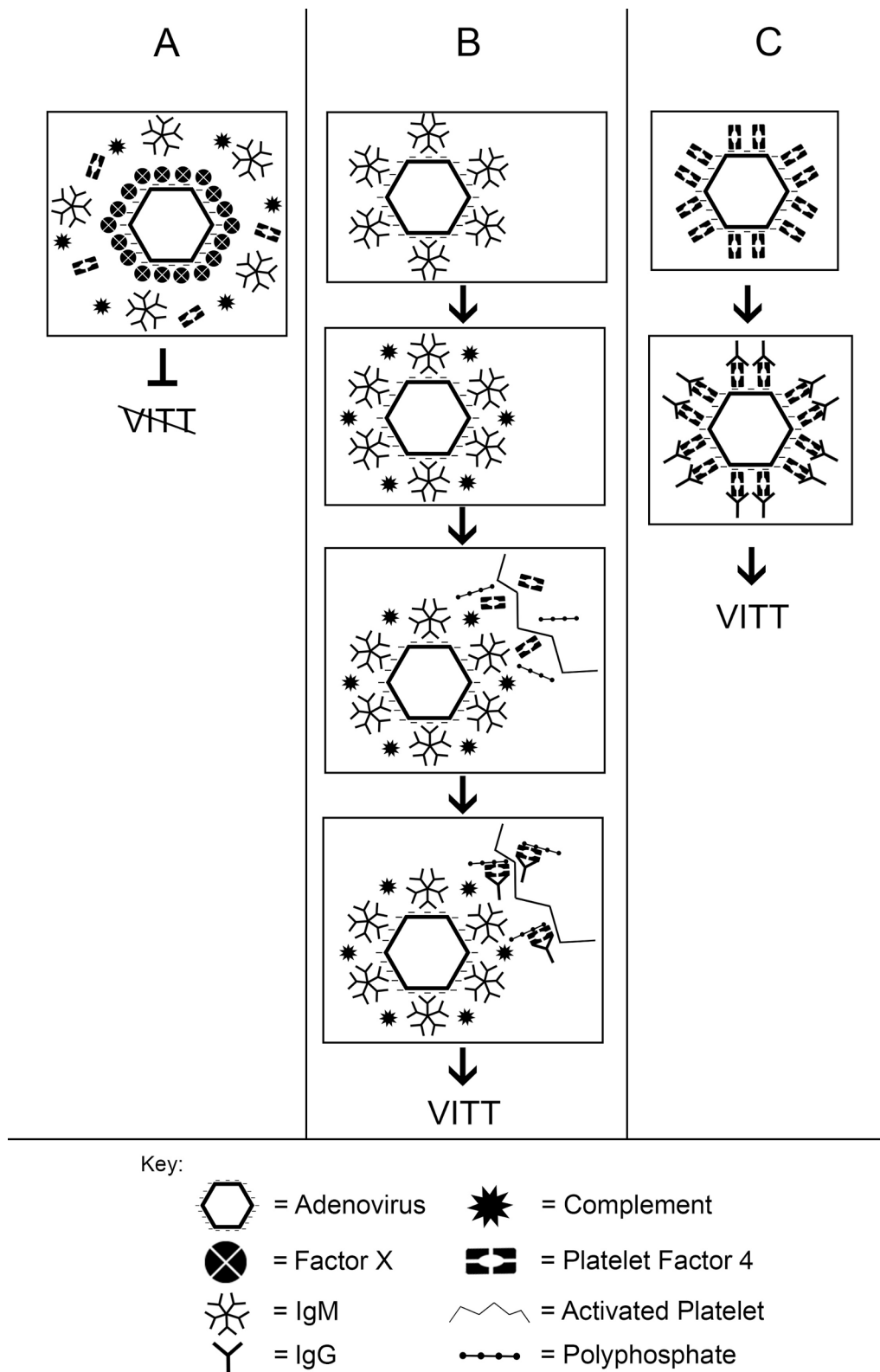


Fig. 1. Schematic of mechanisms for thrombotic response to adenovirus Covid-19 vaccine. Panel A, normal mechanism: adenovirus vector binds endogenous factor X, preventing interaction with IgM, complement and platelet factor 4 (PF4). Outcome: no VITT. **Panel B, adverse mechanism 1:** adenovirus is unprotected by factor X, allowing interaction with IgM, promoting complement activation and activated/aggregated platelet-release of prothrombotic components including PF4 and polyphosphate. PF4-IgG immune complexes are formed. Outcome: up-regulation of coagulation and VITT. **Panel C, adverse mechanism 2:** adenovirus is unprotected by factor X, allowing PF4 binding to the polyanionic (possibly polysulfated) capsid surface. Adenovirus-PF4-IgG complexes are formed, activating complement and platelets. Outcome: up-regulation of coagulation and VITT.

event, requiring large population numbers to identify a possible association with vaccine use.

The mechanism described here proposes that binding of factor X to adenovirus is impaired in a few vaccinated individuals, exposing the unprotected vaccine vector to an immune response which then triggers these rare thrombotic events. Given a similar incidence of VITT in the under-55 age group and in nucleotide mutation rates, this binding impairment may feasibly be due to one or more factor X gene mutations which do not cause clinically-significant coagulation defects. Although such mutations would probably not code for amino acids which significantly influence coagulant activity, the coagulant-active and adenovirus-active binding sites could share common space within the three-dimensional protein structure. The occurrence of differences in factor X primary structure offers the simplest explanation for impaired binding to adenovirus. Studies on human factor X mutations have not been conducted, but factor X from different species has been shown to exert a differential effect on adenovirus transduction via negatively-charged heparan sulphate on cell surfaces [26]; however, the binding affinity of factor X to hexon protein may depend more on adenovirus type than on factor X species sequence [27]. A more complicated mechanism, e.g. involving a putative factor X-binding inhibitor, would need to explain the otherwise normal coagulation status of affected individuals.

The proposed mechanism would anticipate a similar incidence of impaired factor X protection of adenovirus across all age groups. Although this contrasts with the age-stratified *meta*-analysis for VITT incidence, the mechanism remains compatible with these estimates given the different confidence intervals reported for each age range [6] and the potential for age-related differences in the subsequent immune response to exposed adenovirus which leads to VITT.

Depending on the vaccine production conditions, the vector may be: (i) fully-protected by factor X bound during manufacture; (ii) insufficiently saturated with bound factor X during manufacture; or (iii) reliant upon factor X binding after administration to factor X-competent individuals. The proposed mechanism favours the latter condition. Given the blood flow through resting muscle [28] and the tight binding of factor X to hexon protein [13], the circulation in healthy individuals could supply a sufficient excess of endogenous factor X to saturate the adenovirus at the injection site within one minute of the vaccine administration (derivation shown in Table 1). However, for individuals without adenovirus-binding factor X, the vector would remain unprotected and susceptible to immune reaction with IgM and complement

Table 1
Derivation of factor X required for binding to adenovirus.

| Parameter | Value | Formula |
|---|--|------------------------|
| Factor X binding sites per virus particle [13] | 205 | A |
| Assume virus particles per infectious unit | 1 | B |
| Infectious units per 0.5 mL vaccine dose [8,9] | $2.5 \times 10^8 - 1 \times 10^9$ [a] | C |
| Factor X molecules required per dose | 2×10^{11} | $A \times C = D$ |
| Avogadro number | 6.02×10^{23} | E |
| Moles of factor X required per dose | 3.4×10^{-13} | $D \times E = F$ |
| Molecular weight of Factor X [35] | 59,000 | G |
| Grams of factor X required per dose | 2×10^{-8} | $F \times G = H$ |
| Milligram of factor X required per dose | 2×10^{-5} | $H \times 1000 =$ I |
| Specific activity of factor X, IU/mg of protein [36] | 125 | J |
| Units of factor X required per dose | 2.5×10^{-3} | $I \times J = K$ |
| Resting muscle blood flow, L/minute [28] | 2 | L |
| Plasma in blood, L/L | 0.55 | M |
| Plasma concentration of factor X, IU/L | 1000 | N |
| Factor X flow through resting muscle, IU/min | 1100 | $M \times N = O$ |
| Factor X flow per minute excess over dose requirement | 4.4×10^5 | O/K |

[a]subsequent calculations use 1×10^9 infectious units per vaccine dose, assuming equivalence with number of virus particles.

activation/depletion (which is consistent with some case histories [29]). This would then stimulate the thrombotic sequelae described by VITT. Additionally, or alternatively, unprotected sulfated sites on the vector hexon protein may mimic aspects of heparin binding to PF4, forming immunogenic virion-PF4 complexes which induce anti-PF4 antibodies. Either model would explain the clinical symptoms and diagnosis of VITT. This mechanism is consistent with recent preliminary results indicating an association of VITT antibodies with complexes which include the adenovirus [30].

One challenge to this proposed mechanism is that previous environmental exposure to adenovirus infection would already have elicited a thrombotic response in these factor X-compromised individuals before exposure to the Covid-19 vaccine. However, given the multiplicity of adenovirus groups and serotypes and the variable prevalence reported in various adult populations [31,32,33] the number of unexposed individuals remains greater than 1 in 100, which is more than 2 orders of magnitude greater than the estimated incidence of VITT. Although this does not disprove the challenge, it demonstrates that a sufficient proportion of the population would not have had prior exposure to adenovirus for the proposal to remain feasible. Furthermore, even if an individual had prior exposure to adenovirus, the mechanism remains consistent with a hypothesis that VITT is a secondary (typically stronger) immune response following a second (vaccine) exposure to adenovirus [5].

Even if disruption of factor X binding to adenovirus is not due to protein mutation, the proposed root cause mechanism for VITT remains compatible with published evidence about the behaviour of these particular vaccine vectors.

Although the present proposal has focussed on factor X, it is recognised that some adenoviruses can bind factor IX (and possibly other coagulation proteins) [14]. The model would remain valid if different coagulation proteins were substituted, accepting that the binding affinities may vary.

Consequences of the hypothesis

Hypothesis testing

In contrast to the difficulty in identifying a cause for individual adverse events retrospectively, this hypothesis has the potential to be tested by various studies, using established methods (e.g. gene sequencing, immunoassays, immunofluorescent-labelling, light scattering, surface plasmon resonance and cryoelectron microscopy) and available samples, including:

- screening for mutations on the 13F10 gene of affected individuals;
- measuring human factor X binding to the Covid-19 vaccine adenovirus vectors; and
- measuring IgM/complement/PF4 interactions with the Covid-19 vaccine vectors in the presence and absence of human factor X.

Specific experimental controls would be necessary, to avoid potentially-confounding effects of excess factor X and excipients in the reagents and samples.

Preventative action

If this mechanism is proven experimentally, it may be possible to eradicate the hazard by modification of the vaccine manufacturing process to incorporate a binding step for factor X (or an alternative protective agent). Such a step would avoid dependency on endogenous factor X to protect each vaccinated individual against VITT. This should be feasible given the tight factor X binding constant (10^{-9} M or less) [13], equivalent to 2×10^{-8} g (2.5×10^{-3} International Units) of factor X per 0.5 mL vaccine dose (Table 1). The factor X should not be activated, to avoid cleavage of subsequently-expressed SARS CoV-2 spike

protein (previously reported for SARS CoV-1 [34]) which could impair vaccine efficacy. The proposed mechanism predicts that such modification during vaccine manufacture would minimise the risk of thrombotic adverse events in the few at-risk individuals while remaining safe for all other vaccine recipients. The additional factor X in a 0.5 mL vaccine dose would itself exert negligible haemostatic effect, being orders of magnitude less than normal factor X levels in the circulation.

Subject to experimental confirmation, this hypothesis may offer a route to the enhanced safety and utility of adenovirus-vectored vaccines, addressing concerns and facilitating uptake for the benefit of global health.

Declaration of Competing Interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Addendum

Since completing this paper, two studies [37,38] have confirmed the binding of PF4 to the ChAdOx1 adenovirus with an affinity which is at least two orders of magnitude weaker than that of factor X, consistent with the protection proposed by this hypothesis. However, the very low incidence of VITT remains unexplained by these findings, so experiments to test the hypothesis are still needed.

PAF is responsible for development of these mechanistic models and manuscript preparation.

PAF is a consultant of Queen's House Consulting Ltd. and has no competing interest.

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