Chapter 3

Not Only Heparin but Also Antibody Induces Thrombocytopenia

Thi-Huong Nguyen

Additional information is available at the end of the chapter

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Abstract

In the last two decades, heparin was widely used as an anticoagulant. Besides numerous advantages of heparin, some patients with heparin administration suffer from a side effect, the so-called heparin-induced thrombocytopenia (HIT), which can result in thromboses such as deep vein thrombosis, pulmonary embolism, occlusion of a limb artery, acute myocardial infarct, stroke, and a systemic reaction or skin necrosis. The basic on HIT complication have been investigated and led to clinical insights. Recent studies provided detail mechanisms among binding partners in HIT; especially, it has been shown that not only heparin but also a subset of antibody induce thrombocytopenia. In this chapter, insights into both heparin- and antibody-induced thrombocytopenia will be discussed and the novel mechanism of the autoimmune HIT caused by a subset of antibodies will be introduced.

Keywords: heparin-induced thrombocytopenia, HIT, mechanism, binding force, PF4, antibody

1. Introduction

Heparin-induced thrombocytopenia (HIT) as a severe adverse drug effect occurs when patients receive heparin anticoagulant to prevent and treat thromboembolic diseases. Depending on the length of heparin, HIT occurs in ≤5% of patients receiving high molecular weight unfractionated heparin, whereas ≤1% of patients receiving low molecular weight heparin. In HIT, the immune system considers the platelet factor 4 (PF4), which is altered in its conformation after binding to heparin (H), to be "foreign" and the formation of anti-PF4/H antibodies (aPF4/P Abs) occurs. Upon binding to the PF4/H complex, these antibodies activate circulating platelets and other cells. Typically, 5–14 days after heparin exposure, platelet count reduces to <15–20 × 10^9 cells/L (or a > 50% decrease in platelet count). HIT can result

in thromboses such as deep vein thrombosis (DVT), pulmonary embolism (PE), occlusion of a limb artery, acute myocardial infarct, stroke, and a systemic reaction or skin necrosis. Importantly, there is also a subset of anti-PF4/Heparin antibodies (aPF4/H Abs) which, in the absence of heparin, can lead to symptomatic thrombocytopenia and excessive vascular thrombosis. The extreme sequela of the aPF4/H Abs is autoimmune HIT, in which individuals develop multiple vessel occlusions without drug exposure.

2. Heparin-induced thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is a distinct clinicopathologic syndrome caused by platelet-activating antibodies that bridge between complexes of platelet factor 4-Heparin (PF4/H) and platelets [1, 2] or endothelial cells [3]. Human platelets are anuclear cell fragments with discoidal shapes of 1–2 μm, originating from the cytoplasm of bone marrow megakaryocytes [4]. Platelets store PF4 (a positively charged tetramer belonging to CXC chemokine family) in their alpha granules. Non-activated platelets release some PF4s (**Figure 1A**) [5]. When patients take anticoagulant polyanions like heparin, some of these heparins bind to PF4s forming ultra large PF4/H complexes (**Figure 1B**). Binding of heparin to PF4 induces a conformational change in PF4s [6–8] which results in an expression of new epitopes. Some patients develop antibodies against these neoepitopes (**Figure 1B**). These human-derived antibodies are defined as anti-PF4/H antibodies (aPF4/P Abs). Each resulting multimolecular complex

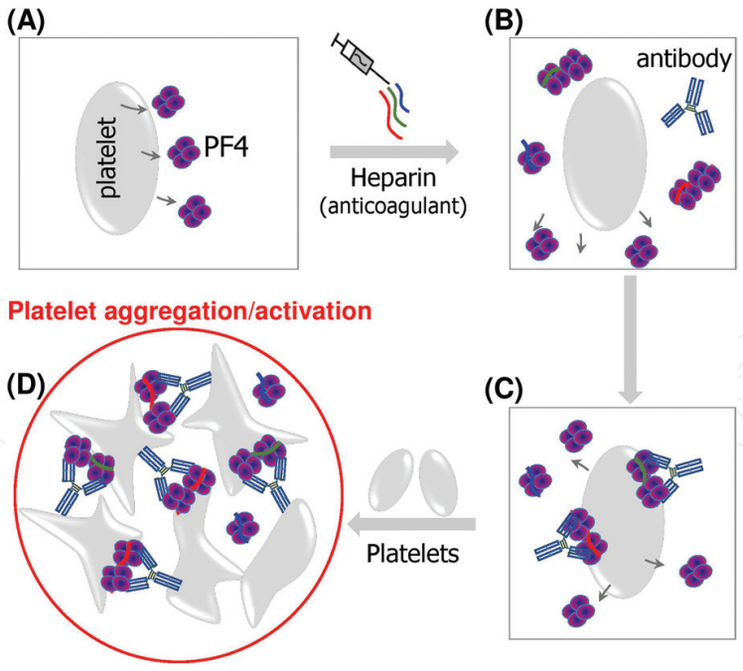

Figure 1. Cartoon illustrates the formation of heparin-induced thrombocytopenia (HIT). (A) Non-activated platelets secrete several PF4s. (B) with heparin exposure, PF4s form ultra large complexes with long heparins that induce conformational changes in PF4s. Some patients develop aPF4/H Abs against PF4 neoepitopes. (C) Human-derived aPF4/H Abs bound PF4/H complexes can adhere to platelet membrane. (D) Fc parts of the antibodies link fcγRIIa receptors on platelet membranes that leads to platelet aggregation/activation. Adapted from [5].

of an aPF4/P Ab to a PF4/H complex contains two platelet binding sites, that is, one is on the PF4/H complexes, and another one is on the Fc part of the IgG which binds to FcγRIIa receptors [9, 10] on platelet membranes (**Figure 1C**). Cross-linking of the platelet Fc receptor results in platelet activation that releases more PF4s and facilitates formation additional ultra large immune complexes. These complexes rapidly recruit other platelets into the prothrombotic process (**Figure 1D**). Activation of platelets leads to the loss of platelets, massive platelet activation and even triggers clotting cascade that results in thrombin generation and increases the risk for vessel occlusions such as venous thrombosis, myocardial infarction or stroke [7, 11, 12]. The binding strength of a blood thrombus has major biological importance. A recent study could determine directly the binding strength between two platelets at single platelet level [13]. The binding force increases proportionally to the degree of platelet activation but reduces with blockade of specific platelet receptors. The method provides major perspectives for testing and improving the biocompatibility of new materials, quantifying the effect of drugs on platelet function, and assessing the mechanical characteristics of acquired/inherited platelet defects.

Heparins are the glycosaminoglycans (GAGs) containing glucosamine residues with a high degree of sulfation that dictates their biological activities [6, 14, 15]. GAGs play an important role in the sequestration of plasmodium falciparum-infected red blood cells in the microvascular endothelium of different tissues [16, 17]. Their pharmacologic activity is mediated by a chemically unique pentasaccharide sequence present in about 30% of all heparin molecules. Heparin behaves like simple entropic spring forces, which is produced by sugar rings of heparin flipping to more energetic and more extended conformations [18, 19]. Both low and high molecular weight heparins are available. The source of high molecular weight unfractionated heparin (UFH) influences the risk of HIT, i.e. bovine UFH is more likely to cause HIT than porcine UFH [20–22]. Besides UFH, the low molecular weight heparins (LMWH) produced from UFH by chemical fractionation, are widely used in clinical practice [23–27]. Due to their shorter chain length, LMWHs show less strong interaction with PF4. UFH and PF4 form ultra large complexes (ULCs) when both are present approximately at an optimal 1:1 ratio. Comparing with UFH, LMWHs form smaller complexes with PF4. ULCs showed a greater capacity to promote platelet activation than small complexes [28]. These differences in complex formation between UFH and LMWHs translate into their risk for inducing HIT in patients. LMWHs induce HIT about 10 times less frequent than UFH, but HIT still randomly occurs during treatment with LMWHs [29–32].

2.1. Boundary between antigenic and non-antigenic heparin

PF4/heparin (or polyanions) complexes can become antigenic or not depend on heparin (or polyanion) characteristics. To expose neoepitopes on PF4s relevant for HIT, at least three bonds between the polyanion and PF4 in the PF4/polyanion complex should be formed [33]. These neoepitopes on the PF4/polyanion complexes then allow binding of the aPF4/H Abs. The binding strength of the single sulfate groups on the polyanion with the PF4 does not differ among polyanions with a different degree of sulfation [33]. The quantity and resulting density of sulfate groups on the polyanion chain determine their molecular effects on PF4 [33]. In particular, the polyanions which bind to PF4 tetramer with less than three sulfate bonds are unable to expose the neoepitope [6, 34]. The results suggest an existence of a boundary between antigenic (risk for HIT) and non-antigenic heparins (non-risk for HIT). This boundary has been determined by

applying multiple techniques such as atomic force microscopy-based atomic force microscopy (AFS) [35], isothermal titration calorimetry (ITC) [6], or circular dichroism (CD) spectroscopy in combination with enzyme-linked immunosorbent assay (ELISA) [7] (**Figure 2**).

AFS shows that both numbers of specific rupture events and magnitude of rupture forces rise with an increase of heparin length, suggesting that long heparins form with PF4 more bonds than short ones [35]. A larger variation of the rupture forces for long heparins ≥8-mer compared with short heparins ≤6-mer was observed (**Figure 3A**). The enthalpy obtained by ITC rises with the increase of heparin length and reaches maximal values at ~11-mer (**Figure 3B**) [36]. Combining the results obtained by AFS and ITC, the boundary between non-antigenic and antigenic heparin is determined between 8- to 11-mer. This boundary is further clarified by CD spectroscopy which is sensitive to the secondary structure and folding properties of proteins [37]. For PF4/H interactions, the change in β-sheet content was found to be ≤30% for short heparin and >30% for long heparins (**Figure 3C**). By ELISA, optical density (OD) was ≤0.5 for short heparin, while OD was >0.5 for longer heparins (>8-mer) (**Figure 3C**). The OD of 0.5 is the threshold to determine whether a heparin used in the ELISA was able to support binding of aPF4/H Abs. The combination of β-sheet content and OD values show clearly a dissimilar behavior between short and long heparins (**Figure 3C**).

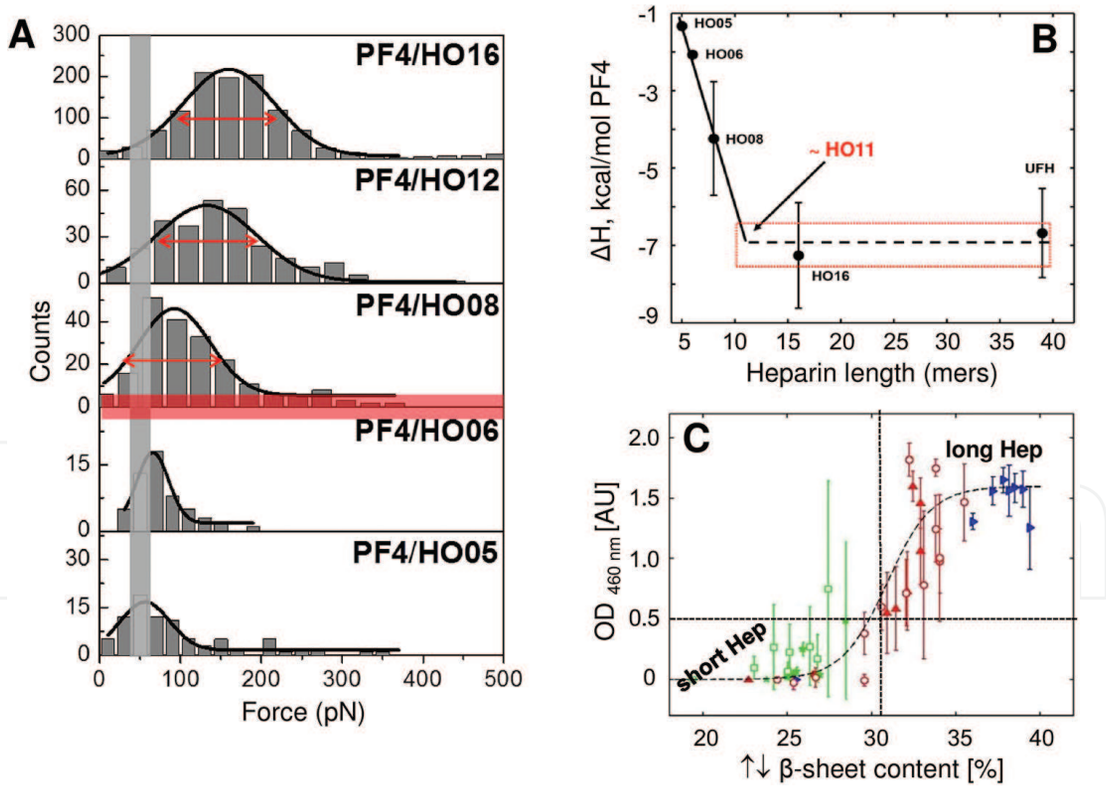

Figure 2. Determination of the boundary between antigenic and non-antigenic heparins. (A) Rupture force histograms fitted by Gaussian distributions show narrow widths (green arrows) for heparins ≤6-mer (HO05, HO06) and wider widths for longer heparins ≥8-mer (HO08, HO012, HO016). (B) ITC demonstrates lower enthalpy for short heparins (black dotted box) and higher enthalpy for long heparins (red-dotted box), while a saturation is found at ~11-mer. (C) Combination of CD spectroscopy and EIA shows that a boundary between short and long heparins is at ~30% ß-sheet contents and OD ~0.5. Overall, the boundary is determined between 8- and 11-mer. Adapted from [8, 35, 48].

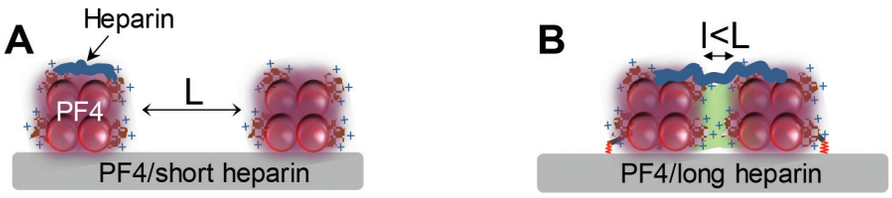

Figure 3. Model describing different binding pathways between short and long heparins when interacting with PF4 tetramers. (A) Depending on heparin length, short heparin can bind to one PF4 tetramer, (B) whereas long heparin bridges two PF4s and forces them closer to each other at a distance l < L, merging two hydrophobic surfaces of PF4s (green shaded area). Adapted from [35].

Linking together all the results from AFS, ITC, CD spectroscopy and ELISA, the boundary between antigenic and non-antigenic heparin has been proved between 8- and 11-mer. These findings are particularly important to understand PF4-Heparin binding processes and to develop new heparin-derived drugs with reduced risk for adverse immune reactions. Combination of these techniques allows better characterizing heparin boundary.

2.2. Kinetic properties and binding model of PF4/H complexes

Thermodynamic and kinetic parameters of the ligand-receptor interactions can be obtained by applying the Bell-Evans [38] or the Friddle [39, 40] models. The models show that the faster the molecule is pulled, the higher the rupture force will be measured. For simple ligand-receptor interaction in which multiple interactions are not involved, the rupture force (F) increases proportionally to the logarithmic loading rate. Even though there is some variation in the parameters obtained by these two models, Bell-Evans model is still a powerful tool to determine the kinetics of ligand-receptor interactions [41, 42]. For the PF4/H system, the PF4 tetramer is considered as one antigen or the interaction between heparin and PF4 is formed by a single bond, and therefore, applicable to the Bell-Evans model [35]. Short heparins show higher k_{off} values than long heparins, indicating that PF4/long heparin complexes are more stable than PF4/short heparin complexes (**Table 1**). With binding affinity (K_A) measured by ITC [6], the thermal on-rate ($k_{on} = k_{off}. K_A$) of PF4/H complexes is calculated. The short heparins bind to PF4s with ~10–20 times faster than long heparins [35].

PF4-Heparins interaction is more complex than general ligand-receptor interactions which are attributed to the electrostatic attraction. Based on special features in force-distance curves and the magnitude of PF4/H binding forces, it has been proved that long heparin bound PF4s creating additional PF4-PF4 bonds [35]. Long heparins form two types of bonds with PFs, i.e.

Parameter	HO06	HO12	HO16
k_{off} (s^{-1})	1.64	1.40×10^{-2}	1.10×10^{-4}
k_{on}(M^{-1} s^{-1})	0.41×10^{5}	0.32×10^{4}	0.55×10^{3}
ΔE ($k_B T$)	−0.49	4.27	9.12

Table 1. Thermodynamic and kinetic parameters of PF4/heparin interactions [35].

(i) PF4-Heparin and (ii) PF4-PF4 bond, whereas short heparins form only one PF4-Heparin bond. Even though the concept of the PF4-PF4 bond, in general, cannot be accepted because PF4s are highly positive proteins, and therefore, strongly repel each other. However, when forming a complex with a highly negative charged heparin, the positive-charged PF4 is probably neutralized that results in a mergence of two hydrophobic PF4 surfaces [34]. Based on these findings, a model for PF4-heparin interaction has been proposed (**Figure 3**). Due to their sizes, the short heparins simply bind to a single PF4 tetramer (**Figure 3A**), whereas the long heparins neutralize positive charges on PF4 tetramers and switch the charges between two PF4 tetramers from a repulsion to an attraction. Heparin reacts as a catalyst that forces two PF4 molecules close to each other within a distance l ($l < L$), resulting in two merged hydrophobic PF4 surfaces (**Figure 3B**). This way of interacting results in the extremely stable PF4/H complexes, especially for long heparins.

A sequence in the formation of PF4/heparin complexes has been identified. When a long heparin comes closely to PF4s, heparin forms first bonds with positively charged clusters on PF4s and then it pulls closely PF4s together to form PF4-PF4 bonds [35].

Based on bond energy (ΔE), quantitative information of bond transitions can be calculated following the study of Wang et al. [43]. The bond transitions of short heparin from the weak positive-charged area on PF4 release energy, whereasPF4-PF4 bonds consume energy [35]. In contrast to short heparin, the bond transitions of long heparins in both cases release energy, while their interactions with the positively charged clusters consume energy (**Table 1**). Based on energy level, PF4-PF4 interaction is attributed to be stronger than the bonds between heparin and non-clusters of positive-charged areas on PF4. However, PF4-PF4 interaction is weaker than the interaction between heparin and clusters of positive charges on PF4.

3. Antibody-induced thrombocytopenia

Immunocomplexes composed of aPF4/P Abs and PF4/polyanion (PF4/P) complexes on the platelet surface induce platelet aggregation via cross-linking FcγRIIA receptors [9, 10]. They also bind to the surface of endothelial cells and monocytes [44–46], inducing procoagulant activity [44, 47]. Heparin-induced thrombocytopenia has been well understood. Recent studies reported that a subset of human-derived autoantibodies in some patients also can induce thrombocytopenia in a heparin-similar manner.

3.1. Human-derived HIT antibodies

All aPF4/P Abs bind to immobilized PF4/P complexes in ELISA [48], but only some of them activate platelets in functional assays, e.g. the heparin-induced platelet activation assay (HIPA) [48] or the serotonin release assay (SRA) [49, 50]. Human-derived aPF4/P Abs compose of three groups, i.e. the antibodies do not activate platelets in HIPA test (group-1 Abs); the antibodies activate platelets in HIPA but require heparin (group-2 Abs); the antibodies activate platelets even without heparin (group-3 Abs) (**Figure 4**). Group-3 Abs developed from patients who had clinical autoimmune HIT, and therefore, they are defined as 'autoantibodies' [51].

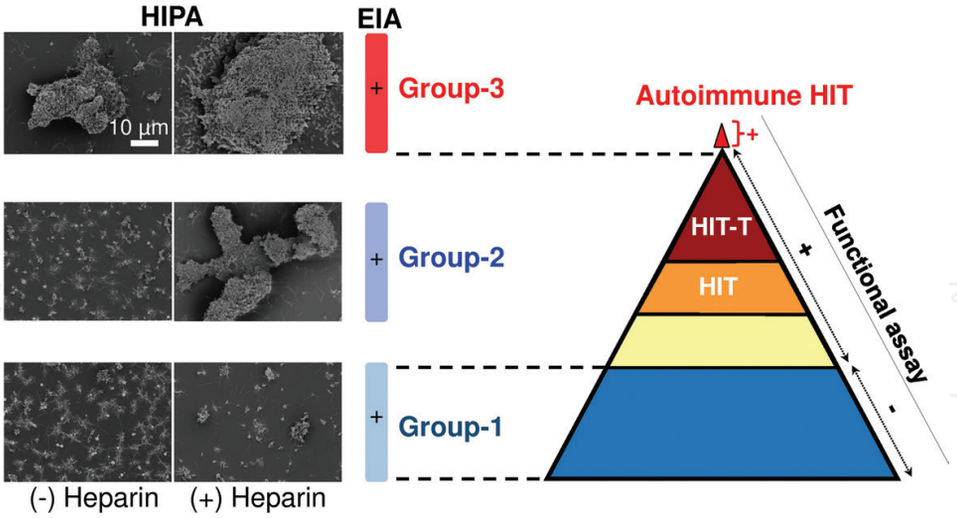

Figure 4. Different reaction patterns of aPF4/H antibodies. (Right) pyramid shows antibodies of three groups, all positive in EIA. Group-1 (blue) do not activate platelets (HIPA -); many Abs belonging to group-2 do not induce HIT (yellow), some induce HIT (gold) and others induce HIT with thrombosis (dark red). Recent studies found an additional small subset of patient's content autoimmune group-3 HIT Abs (red). (Left) visualization of platelet aggregates-induced by different antibody groups imaged by scanning electron microscopy in the presence (+) or absence (−) of heparin: Group-1 abs induce (bottom left) only small aggregates reflecting the background platelet activation; group-2 Abs (middle, left) cause large aggregates only in the presence of heparin; group-3 Abs induce large aggregates even in the absence of heparin. Same scale bar for all images. Adapted from [55].

3.1.1. Characteristics of human-derived HIT antibodies

In contrast to the detailed characterization of the PF4/polyanion complexes, little is known about the features of aPF4/H Abs in the pathogenesis of HIT. Exploring the characteristics of HIT antibodies bears a potential to better understand general mechanisms of antibody-mediated autoimmunity HIT. However, there is a difficulty in subtracting the pathogenic HIT antibody directly from human sera because both pathogenic and non-pathogenic antibodies bind to the PF4/H antigen.

Newman et al. reported that aPF4/P Abs can be purified by PF4-agarose beads [3]. Later in 2000, Amiral et al. described that affinity purification of aPF4/P Abs resulted in a mixture of IgA, IgM, and IgG [52]. In this mixture, only a subset of IgG antibodies activates platelets [49]. Contamination of IgA, IgM, and IgG antibodies will increase the difficulty in characterizing aPF4/P Abs. To overcome this limitation, two-step affinity chromatography has currently established to separate aPF4/H Abs from HIT patients sera. By this method, aPF4/P Abs from sera of patients were successfully isolated for three antibody groups. The purified Abs showed similar characteristics as the original serum in EIA and HIPA. Titrating the antibodies in ELISA, all antibody groups show an increase of OD with increasing antibody concentration (**Figure 5A**). OD values are highest for group-3, followed by group-2 and then group-1 Abs. In the HIPA test, group-1 Abs did not cause platelet aggregation up to a concentration of 89.7 µg/mL; group-2 Abs induced platelet aggregation from concentrations ≥43.5 µg/mL, but only in the presence of heparin; while group-3 Abs induced platelet aggregation from concentrations ≥5.2 µg/mL independently of heparin (**Figure 5B**). This is consistent with previous findings that chondroitin sulfate plays an important role in platelet activation by PF4/P Abs, even in the absence of heparin [53, 54].

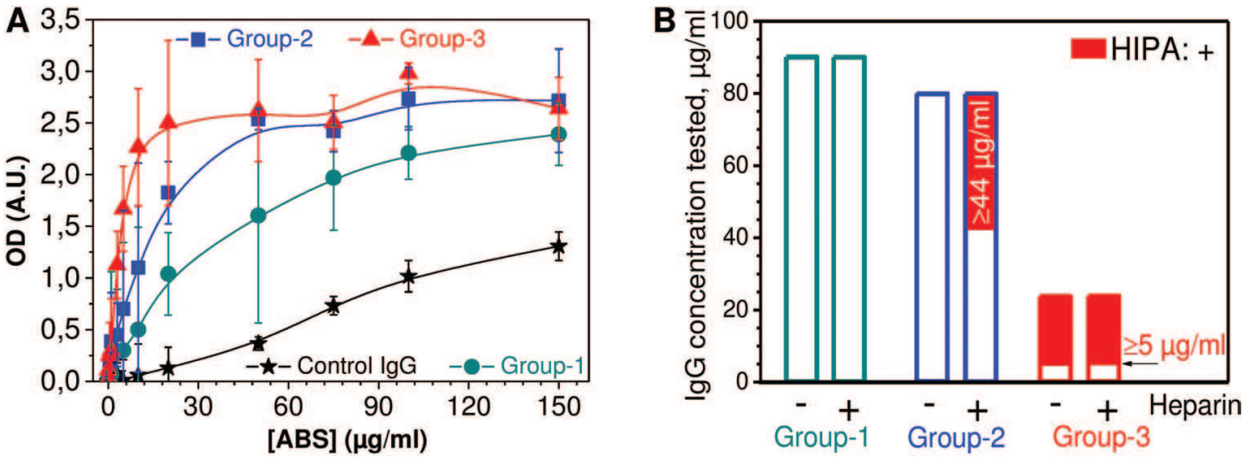

Figure 5. Dose-dependent binding of aPF4/P Abs to PF4/H complexes in EIA and HIPA. (A) EIA shows the lowest OD of control IgG (black) as the background reaction, follow by group-1 (dark cyan), higher for group-2 (blue) and highest for group-3 (red) Abs. (B) HIPA tests show a dependence of platelet aggregation on antibody concentration: Group-1 Abs do not activate platelets, neither in the absence (−), nor in the presence (+) of reviparin up to a concentration of 89.7 µg/mL (dark cyan); group-2 Abs (blue) induced platelet activation (red part) at concentrations ≥44 µg/mL but only in the presence of reviparin; group-3 Abs (red) activated platelets at much lower concentrations (≥5 µg/mL) either in the presence or absence of reviparin. n = 5 sera per group. Adapted from [55].

3.1.2. Binding strength of human-derived HIT antibodies

The binding strength between the antibody and PF4/H complexes is determined by AFS. A single aPF4/H Abs is immobilized on the cantilever and then approach to the PF4/H complexes coated on a solid phase for interacting and measuring of their binding strength. Weakest binding forces were measured for monoclonal antibody KKO mimicking human HIT antibodies (43.6 ± 8.8 pN, gray) and group-1 Abs (44.0 ± 8.1 pN, green), higher for group-2 Abs (60.6 ± 15.4 pN, blue) and highest for group-3 Abs (72.4 ± 26.2 pN, red). Statistics showed no significant difference between KKO and group-1 Abs ($p = 0.877$), significant difference between group-1 and group-2 Abs ($p < 0.001$), or between group-2 and group-3 Abs ($p = 0.006$)) (**Figure 6**) [55].

Group-3 Abs bound to PF4/H complexes with much higher binding energy ($\Delta H = -2.87 \pm 2.06 \times 10^8$ cal/mol) than group-2 Abs ($\Delta H = -2.90 \pm 0.4 \times 10^4$ cal/mol), and their dissociation constant (K_D) (~5.3 nM) was about two orders of magnitude lower than that of group-2 Abs (~1.7×10^2 nM). The binding strength of PF4 to heparin ~150 pN [35] is higher than that between group-3 Abs and PF4/H complexes (mostly lower than 150 pN) [55]. Besides that, the group-3 Abs have a highest binding affinity ($k_{off} = 0.12\ s^{-1}$) as compared with group-1 Abs y ($k_{off} = 15.6\ s^{-1}$), group-2 Abs ($k_{off} = 2.0\ s^{-1}$), or KKO ($k_{off} = 2.2\ s^{-1}$). The lowest thermal off-rate specify that multiplexes induced by PF4/H complexes with group-3 Abs are more stable than those formed with other antibody groups. Furthermore, KKO and group-1 Abs contain antibodies with similar characteristics, and therefore, they interacted rather uniformly with PF4/H complexes. This has been clarified by obtaining the relatively small differences among the rupture forces (<60 pN, **Figure 6A-B**) measured from different cantilevers. However, group-2 Abs contain different types of antibodies as observed by a large variation of all binding forces (~40% exceeded 60 pN). For group-3 Abs, the variation of binding force is even higher than that of group-2 Abs as shown by ~44% of all binding forces ≥60 pN and ~15%

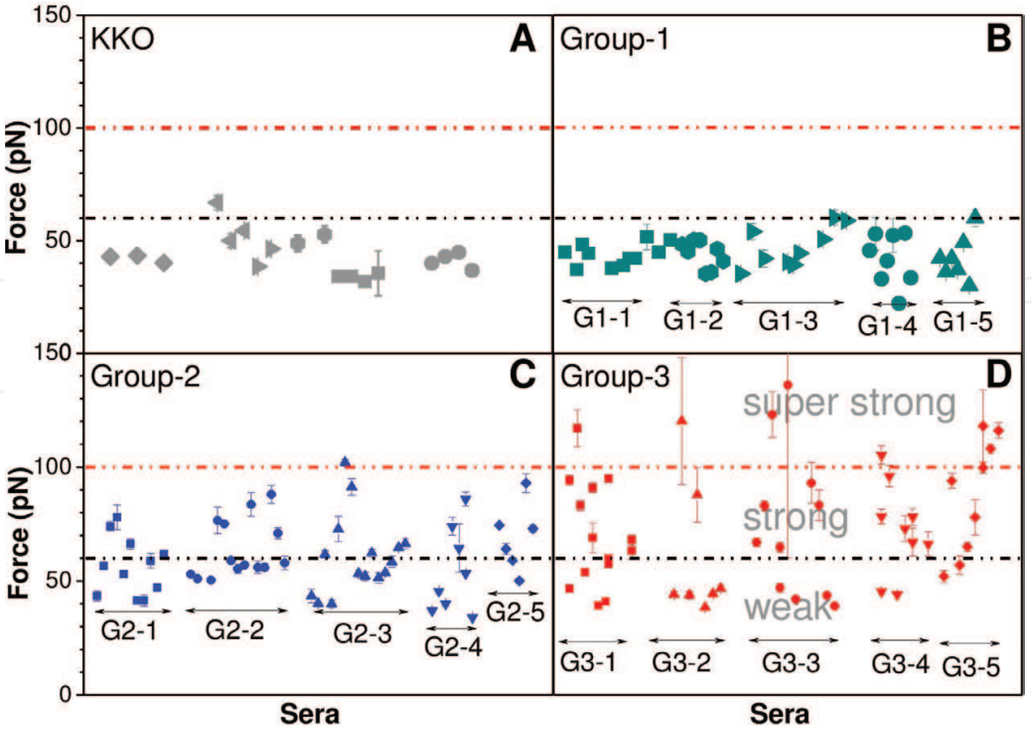

Figure 6. Binding characteristics of aPF4/H Abs. Each dot shows the mean and standard error of the rupture force for each respective antibody from five sera per group. (A) KKO and (B) group-1 Abs bind to PF4/H complexes with a binding strength mostly ≤60 pN (black dotted line), while (C) group-2 and (C) group-3 Abs consist of Abs with different binding forces. (D) a subset of group-3 Abs binds to PF4/H complexes with rupture forces higher than 100 pN (red-dotted line). Adapted from [55].

even exceeded 100 pN. The low variability in binding forces of KKO and group-1 Abs has been attributed to the fact that they contain homogeneous antibodies, whereas the patient's sera such as group-2 and group-3 Abs contained polyclonal mixtures of aPF4/P Abs differently reactive. Among these human-derived Abs, it has been proved that the group-2 contains also antibodies reacting like group-1 Abs, while group-3 is highly complicated as it composes of not only antibodies reacting like group-1 and group-2 Abs but also some additional super strong reactive antibodies. The aPF4/H Abs show different reactivity patterns under various pH and ionic strength conditions [56].

3.1.3. Autoimmune antibodies cluster PF4

The autoimmune group-3 Abs activate platelets in the absence of polyanions because they can self-cluster PF4 to form PF4/group-3 antibody complexes without the need of heparin [55]. This characteristic of autoimmune group-3 Abs has been proved by various methodologies:

First, the autoimmune group-3 Abs could be purified from the patient's sera using a PF4-column (instead of the PF4/H column). Hardly any PF4/P Abs were obtained from control and group-1 sera; group-2 sera showed a minimally increased IgG yield. When these antibodies are concentrated to 50 μg/ml, only antibodies purified from group-3 sera activated platelets in the HIPA. The results indicate that group-3 sera contain antibodies with PF4 specificity, which activate platelets.

Next, only autoantibodies (group-3) show strong interaction with PF4 alone by ITC. When the antibodies were tested at the same concentration of 62.5 nM, KKO and group-2 Abs did not interact with PF4, while group-3 Abs interacted strongly. As the interaction between group-3 Abs and PF4 alone showed two binding sites (stoichiometry $n = C_{ABS}/C_{PF4} = 0.53 \pm 0.003$), these Abs can cluster two PF4 molecules. Increasing antibody concentration did not improve binding of KKO to PF4 whereas group-2 Abs weakly interacted with PF4. However, the binding energy released by group-2 Abs is only 0.1% compared to that of group-3 Abs.

Consistently, PF4 or PF4/H EIA show that group-3 Abs bound quite strong to PF4 while other antibodies did not even though all Abs bound much stronger to PF4/H complexes than to PF4 alone. By AFS, group-1 and group-2 Abs showed much less binding events to PF4 than to PF4/H complexes, while the super-reactive group-3 Abs showed similar bindings. In addition, the interaction forces of group-3 Abs purified via a PF4-column with PF4/H complexes showed the highest range of binding forces (~100 pN). These results again indicate that group-3 Abs bind strongly to PF4 alone independently from heparin, while bindings of group-1 and group-2 Abs are heparin-dependent.

By dynamic light scattering (DLS), group-3 Abs formed the largest complexes with PF4 as compared to other antibody groups with even larger size than PF4/H complexes further indicate that group-3 Abs can cluster PF4.

The binding energy generated by the interaction of group-3 Abs with PF4 in the ITC experiments ($\Delta H = -3.5 \pm 0.86 \times 10^7$cal/mol) is much higher than the energy released when a 16-mer heparin interacts with PF4 ($\Delta H = -7.26 \pm 1.36 \times 10^3$ cal/mol) [6]. As 16-mer heparin can force two PF4 molecules together, based on their high energy release, group-3 Abs most probably also can force two PF4 tetramers together. In addition, the negative entropy of the reaction ($\Delta S = -11.7 \pm 2.8 \times 10^4$ cal/mol. K) is attributed to PF4 conformational change when forming complexes with the group-3 Abs. By DLS, the size complexes formed by PF4 and group-3 Abs increases significantly when the group-2 Abs are added, indicating that group-3 Abs, induce a conformational change in PF4 and the resulting PF4/group-3 antibody complexes allow binding of group-2 Abs in the same way as polyanions do.

Altogether, PF4 form large complexes with heparin and allow group-2 Abs bind and induce platelet aggregation/activation (**Figure 7A-C**). Importantly, a subset of group-3 Abs cluster PF4 and the resulting PF4/Group-3 antibody complexes also allow binding of group-2 Abs and enhance platelet aggregation/activation even stronger than heparins do as shown by tighter and denser aggregates (**Figure 7D-F**).

3.2. HIT-like antibodies

Many studies in HIT have been performed with human aPF4/P Abs isolated from patient plasma because only one monoclonal antibody (KKO) mimicking human HIT antibodies did exist until recently [57]. KKO activates platelets [58] and monocytes [59] *in vitro* and *in vivo* by cross-linking FcγRIIa. KKO has been used to unravel the pathogenesis of HIT and is the basis for a recently FDA approved plasma-based antigen assay (HIT-HemosIL) for detection of PF4/P antibodies [60, 61]. KKO mimics the biological activity of human aPF4/P Abs [62]

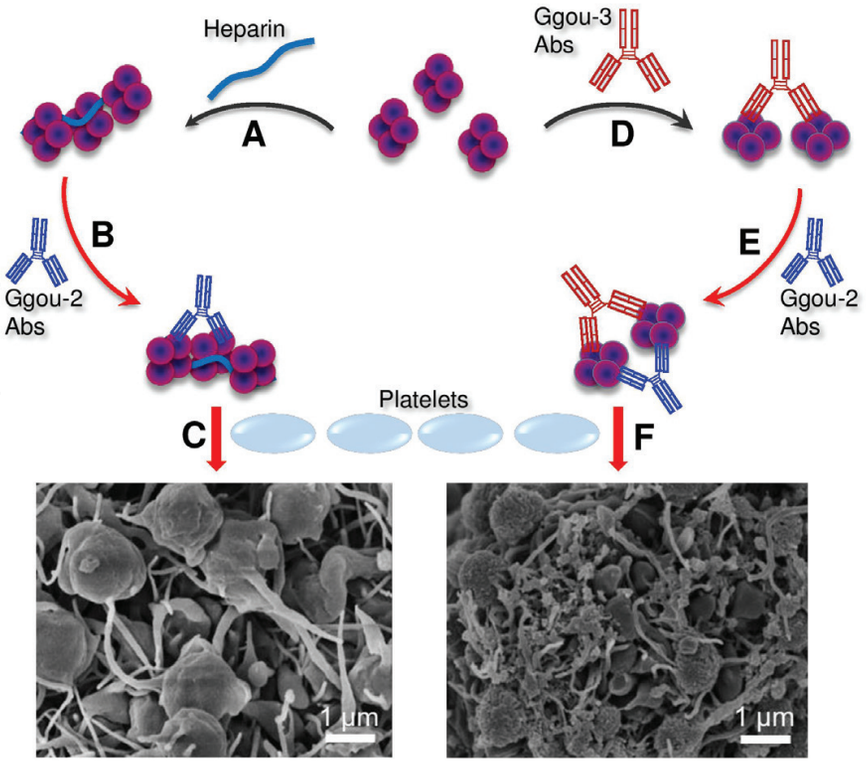

Figure 7. Group-3 Abs cluster PF4 and enhance platelet activation. (A) PF4 form large complexes with heparin and the resulting PF4/H complexes allow (B) group-2 Abs bind and (C) induce platelet aggregation/activation. (D) a subset of group-3 Abs cluster PF4 forming PF4/Group-3 antibody complexes which also (E) allow binding of group-2 Abs and (F) enhance platelet aggregation/activation evidenced by tighter and denser aggregates compared to (C). Adapted from [55].

and has been used to understand the binding characteristics of an antibody recognizing PF4/P complexes and activating platelets [62, 63]. Binding of a non-HIT antibody RTO to PF4 monomers prevents PF4 tetramerization and inhibits KKO and human HIT IgG-induced platelet activation/aggregation in vitro, and thrombus progression in vivo. The probability and the interaction force of KKO binding to PF4 are much greater than those of RTO, while KKO/PF4 dissociation rate was approximately 10-fold slower than RTO/PF4 [62, 63], indicating that KKO binds stronger than RTO and KKO/PF4 complexes are more stable than RTO/PF4.

KKO interacts with PF4/H complexes coated platelets with ~4-fold higher forces than with PF4/H complexes coated on a solid phase, while RTO shows only a minor change [64]. The different binding forces strongly indicate that PF4 and PF4/H complexes either expose different epitopes or allow better access of platelet-activating Abs to their epitope when PF4 bound to the platelet surface compared to the presentation of PF4/H complexes on a solid phase. Most probably, PF4/H complexes exhibited the antigenic site differently depending on the bound substrates [53]. The findings provide an explanation for the surprising observation that KKO interact relatively weak when PF4/H complexes are immobilized on a solid phase [55], while it strongly activates platelets in functional assays. It is unresolved, which additional binding partners on the platelet surface interfere with the conformational change or different presentations of PF4/H complexes. Nevertheless, chondroitin sulfate [53] and polyphosphates [65] are potential candidates, as they interact with PF4.

However, KKO is a mouse IgG2b antibody (an absent subclass in humans) [66], while the platelet-activating aPF4/P Abs present in HIT plasma samples are predominantly IgG1. KKO behaves differently from human aPF4/P Abs, i.e. it binds only weakly to PF4/H complexes coated on a solid phase [64]. Recently, a chimeric monoclonal aPF4/H Abs with a human Fc fragment (5B9) has been developed [67]. The 5B9 antibody has been demonstrated to fully mimic the cellular effects of human HIT Abs [10, 68].

4. Diagnosis of HIT

Immunologic assays, such as polytypic ELISA, IgG-specific ELISA, and particle gel immunoassay (PGI) have a sensitivity, are widely used to detect aPF4/H Abs in the diluted human sera because of their high sensitivity (≥95%) and the fast turn-around. However, only ~50% of aPF4/H Abs detected by antigen tests are clinically irrelevant. The results from positive immunologic assays may lead to an overtreatment for HIT that can result in serious consequences, such as venous limb gangrene or fatal hemorrhage [69]. However, immunologic assays are still powerful tools to rule-out patients with HIT. The cut-off optical intensity (OD) in ELISA was defined at 0.5. An ELISA test showing OD > 0.5 is normally suspected to contain aPF4/H Abs. To increase the specificity of clinically relevant antibodies, a higher OD cut-off for the antigen tests (e.g. OD > 1.0) had been suggested [70].

Even though functional assays such as by serotonin release assay (SRA) [71] or HIPA [72] have a sensitivity of ~90% which is slightly lower than the immunologic assays, these tests show a much better specificity of over 90%. For the better identifying HIT, it is recommended that a positive PF4/H ELISA should prompt confirmatory testing by functional assays [73]. However, the functional assays are only available in specialized laboratories and not available in many countries. Therefore, many physicians rely on the results of antigen tests, especially for the first days after clinical suspicion of HIT has been raised until the results of the functional assay is reported.

Besides immunologic assays and functional assays, the chemiluminescent immunoassays such as HemosIL AcuStar HIT-IgG and HemosIL AcuStar HIT-Ab have been recently introduced. These methods are relatively faster (~30 minutes) than the immunologic assays (hours) and showed extremely high sensitivity (~100%) [74]. The assays seem to be ideal for ruling out HIT. Another study used a colorimetric test to detect HIT based on the interaction between platelets and tetrazolium-based indicator dye [75]. The authors reported the quality of detecting HIT is from 96 to 100% agreement with the functional assay C-SRA.

5. Conclusion

Not only heparin but also autoimmune antibodies induce thrombocytopenia. Large antigenic complexes formed between PF4 and either heparin or antibody activate platelets, cause a prothrombotic and result in a variety of thromboembolic and systemic consequences. In autoimmune HIT, aPF4/P Abs activate platelets in the absence of heparin. These antibodies are highly reactive. They can self-cluster PF4-molecules forming antigenic complexes and allow

binding of otherwise aPF4/P Abs. The resulting immunocomplexes induce massive platelet activation in the absence of heparin. The source and length of heparins play an important role in inducing thrombocytopenia. Improvement of heparin quality together with discovering new non-heparin drugs should be highly desirable. Patients who are suspected of HIT need to be immediately stopped heparin exposure and switched to an alternative anticoagulant. Regarding patients with antibody-induced thrombocytopenia, the level of complication is much higher than the general heparin-induced thrombocytopenia. To date, these human-derived antibodies are hardly controlled, and therefore, efforts in the field would be appreciated. Clinical tests for detecting HIT antibodies as well as autoimmune HIT antibodies must be improved to achieve an appropriate identification of clinical HIT patients.

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Conflict of interest

The authors declare no competing financial interests.

Author details

Thi-Huong Nguyen

Address all correspondence to: nguyent@uni-greifswald.de

Institute of Immunology and Transfusion Medicine, University Medicine Greifswald, Greifswald, Germany

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