Characterization of new Monoclonal Anti-PF4/Polyanion Antibodies as useful Tools for Studies on Typical and Autoimmune HIT

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ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) is typically caused by platelet-activating IgG antibodies (Abs) against platelet factor 4 (PF4) complexed with heparin (H). Much less frequent "autoimmune" HIT is distinguished from typical HIT by platelet activation without heparin and presence of both anti-PF4/H and anti-PF4 IgG. We developed three murine monoclonal anti-PF4 Abs with a human Fc-part, 1E12, 1C12, and 2E1 resembling autoimmune HIT Abs.

Objectives: To characterize 1E12, 1C12, and 2E1 in comparison to the heparin-dependent monoclonal anti-PF4/H Abs 5B9 and KKO, and polyclonal Abs from patients with typical HIT (group-2) and auto-immune (group-3) HIT.

Methods: Interactions of Abs with PF4 and PF4/H were studied by ELISA, single-molecule force spectroscopy, isothermal titration calorimetry, and dynamic light scattering. Serotonin release assay and heparin-induced platelet activation assay were used to assess platelet activation. The binding sites of monoclonal Abs on PF4 were predicted *in-silico* (MAbTope method).

Results: 1C12, 1E12, and 2E1 displayed higher affinity for PF4/H complexes than 5B9 and KKO, comparable to human group-3 Abs. Only 1C12, 1E12, 2E1, and group-3 Abs formed large complexes with native PF4 and activated platelets without heparin. The predicted binding sites of 1C12, 1E12, and 2E1 on PF4 were close to each other, and differed from those of KKO and 5B9. 2E1 shows unique bivalent binding, with its antigen recognition site to PF4 and in addition charge related to heparin.

Conclusions: 1C12, 1E12, and 2E1 are tools for studying the pathophysiology of autoimmune HIT. 2E1 provides evidence for a new binding mechanism of HIT antibodies.

KEYWORDS: platelet factor 4, monoclonal antibodies, heparin, thrombocytopenia, autoimmunity.

INTRODUCTION

Heparin-induced thrombocytopenia (HIT) is an adverse drug effect caused in most patients by platelet-activating IgG antibodies (Abs) directed against complexes of platelet factor 4 (PF4) bound to heparin (H) [1]. Binding of PF4 to polyanions (P) induces its conformational change [2] and exposes the binding site(s) for anti-PF4/polyanion antibodies (aPF4/P-Abs). This results in a spectrum of symptoms in patients who develop these antibodies ranging from asymptomatic serum positivity for aPF4/P-Abs, over heparin-dependent HIT, to life-threatening autoimmune HIT [3,4].

Major progress has been made in understanding the pathogenesis of HIT in recent years [2,5,6]. However, mechanistic studies with human antibodies obtained from patients have limitations as these sera always contain a polyclonal mixture of antibodies with different binding characteristics [7]. Purification of antibody subgroups from these sera is possible but requires major efforts [6]. Therefore, the availability of well-characterized monoclonal antibodies will likely allow a wide variety of new experimental approaches.

To date, only one murine monoclonal aPF4/P antibody, KKO that mimics the biological function of human HIT antibodies, is commercially available. KKO activates platelets and monocytes by cross-linking FcγRIIa, but it is an IgG2b antibody with an Fc-fragment, which differs from those of human IgG [8]. Also, the binding characteristics of the $F(ab')_2$-part of KKO differ from the ones evidenced with human HIT IgG [9]. More recently we developed 5B9, a monoclonal antibody to PF4/H complexes with a human Fc-fragment, and which fully mimics the cellular effects of human heparin-dependent HIT Abs [10].

It also became obvious that HIT can sometimes present as an autoimmune disease [4], and affected patients develop aPF4/P Abs that activate platelets even in the absence of heparin or polyanions. These autoimmune HIT antibodies can provoke the development of multiple

vessel occlusions without any drug exposure [11]. However, until now no standardized antibody for studying the mechanisms involved in autoimmune HIT was available.

To further study the pathophysiology of various clinical presentations of HIT, we developed new murine monoclonal aPF4/P antibodies (moAbs), 1E12, 1C12, and 2E1, which also have a human Fc-fragment and appear to mimic autoimmune HIT Abs. We utilized our immunological and biophysical methodologies [6] to define their characteristics, compared to those of KKO, 5B9, and different groups of polyclonal aPF4/P Abs isolated from immunized patients, with typical HIT and autoimmune HIT.

MATERIAL AND METHODS

Production of monoclonal antibodies to PF4 and PF4/P complexes

The four monoclonal antibodies (5B9, 1E12, 1C12 and 2E1) have been obtained as previously described [10]. Briefly, transgenic Balb/c mice homozygous for the C gene of human immunoglobulin G (IgG) that directly produce chimeric IgG1 antibodies (GammaPrim^{TM}, B Cell Design, Limoges, France) were injected intraperitoneally with preformed PF4/heparin complexes (human PF4; 50 µg, Hyphen BioMed) and unfractionated heparin (UFH 2 IU/ml; Heparin Choay; Sanofi). Mice were sacrificed and their spleens were removed for fusion procedures, hybridomas were cultured for 13 days, and supernatants were screened by a homemade ELISA, adapted from Arepally et al. [8] using microplates coated with purified human PF4 (2.5 µg/mL, Hyphen Biomed, Neuville-sur-Oise, France) with or without 0.05 IU/mL heparin. Four clones were isolated: three producing antibodies (1C12, 1E12 and 2E1) that equally bound PF4/P complex and PF4 alone, and one that was mainly specific to PF4/P (5B9). These antibodies were isolated from ascitic fluid by using protein G columns (GE

healthcare). The murine anti-PF4/P antibody KKO was purchased from Thermo Fischer Scientific (Les Ulis, France).

Human antibodies to PF4/P complexes

Human anti-PF4/P antibodies were purified from patients as previously described [6], and classified into 3 groups according to their variable ability to activate platelets: group-1 Abs did not activate platelets (negative heparin-induced platelet activation test, HIPA), group-2 Abs induced positive HIPA results but only in the presence of heparin and were considered as typical HIT antibodies, whereas group-3 Abs activated platelets even in the absence of heparin and were therefore defined as 'autoantibodies'.

ELISA for anti-PF4/P moabs

The reactivity of purified KKO, 5B9, 1E12, 1C12 and 2E1 against PF4/P complexes was evaluated by using the commercial IgG-specific ELISA kit Asserachrom® HPIA IgG (Stago, Asnières, France) according to the manufacturer's recommendations, except that we used a peroxidase-conjugated goat anti-mouse IgG light chain antibody as a secondary antibody (Jackson ImmunoResearch, Ely, United-Kingdom). Binding of these antibodies to PF4 alone and to protamine sulfate, or protamine sulfate/H complexes was also assessed as previously described [12], using the peroxidase-conjugated goat anti-mouse IgG light chain.

ELISA for human anti-PF4/P Abs

PF4/H complexes were pre-formed by a mixture of 20 µg/mL PF4 with 0.5 IU/mL heparin (Heparin-Natrium-25,000, Ratiopharm GmbH, Ulm, Germany) in PBS for 1h at RT and

immobilized on a microtiter plate overnight at 4°C [13]. Then, purified human antibodies at different concentrations were incubated with PF4/P complexes for 1h at RT. After washing (150 mM NaCl, 0.1% Tween 20, pH 7.5), wells were incubated with peroxidase-conjugated goat anti-human IgG (1:20 000, Dianova, Hamburg, Germany) for 1h at RT. Bound antibodies were subsequently detected by measuring the optical density (OD) at a wavelength of 450 nm.

Platelet activation and aggregation tests

Whole blood from healthy donors was collected in acid-citrate-dextrose supplemented with prostaglandin E1 (0.1mM, Sigma-Aldrich, Saint-Quentin Fallavier, France) [14]. Platelets were washed and suspended at a final count adjusted to $300x10^6$/mL. Serotonin release assay (SRA) was performed as described [7], with different concentrations of KKO, 5B9, 1E12, 1C12 or 2E1, with or without unfractionated heparin (UFH; Heparin Choay, Sanofi-Aventis, Gentilly, France). For Heparin-Induced Platelet Activation (HIPA) assay, 75 µL of washed platelets were incubated with 20 µL purified antibodies groups 1-3 of 50 µg/ml with the low molecular weight heparin (reviparin) 0.2 aFXau [15]. High heparin concentration (100 IU/mL) was added to inhibit platelet aggregation as a control.

SMFS experiments

SMFS experiments were carried out as previously described [6]. To immobilize antibodies on an AFM-tip, gold-coated silicon nitride cantilevers with a nominal spring constant of 6 pN/nm (Olympus Biolever, Tokyo, Japan) were coated with thiol-PEG-COOH (Mw 3400 Da, Nanocs, USA) and the -COOH group at the end of PEG linkers were activated with the amine coupling kit (Biacore, Uppsala, Sweden), containing the mixture of 0.4 M 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS), for binding of proteins [16,17]. The cantilevers were coated with human or monoclonal anti-PF4/P antibodies at a concentration of 70 µg/mL, 30 min, RT and kept overnight at 4°C. Free activated groups on the surfaces were blocked by adding 1.0 M ethanolamine (Biacore, Uppsala, Sweden) for 1h at RT.

For immobilization of PF4 (20 µg/mL) or PF4/H complexes on the substrate, the same protocol as described for coating antibodies on the AFM tip was used. PF4/H complexes were pre-formed by a mixture of 20 µg/mL PF4 with 0.5 IU/mL heparin (Heparin-Natrium-25,000, Ratiopharm GmbH, Ulm, Germany) in PBS for 1h at RT [18]. For immobilization, proteins were kept on the PEG-coated Au surfaces overnight at 4°C.

The measurements were carried out in PBS using JPK NanoWizard 3 (Berlin, Germany) with a setpoint of 200 pN and 1,000 force-distance (F-D) curves per condition at a velocity of 1µm/s [19]. Thermal off-rates (dissociation rates) of the bonds between antibodies and PF4/H complexes were determined by applying the Bell-Evans model to the relation between loading rates and rupture forces, which were measured at tip speeds ranging from 0.01 to 4µm/s [20,21]. The model describes that the rupture force increases proportionally to the natural logarithm of the loading rate during retraction. JPK data processing software (version 4.4.18+) was used to analyze the measured adhesion forces. The mean rupture force values and their corresponding errors were determined by applying Gaussian fits to the data using Origin software (version 9.1).

Isothermal Titration Calorimetry (ITC)

ITC measurements were carried out using a MicroCal iTC200 calorimeter (Malvern Instruments, Malvern, UK). PF4 in PBS 17 µM was added to the sample cell and a solution of moAbs (1.7 µM) or human antibodies (62 nM) was loaded into the injection syringe. The

antibody solution in the syringe was added by 19 injections of 2 µL each into PF4 in the cell at 25°C, 1,000 rpm stirring with 240 seconds intervals. The one set of sites model was used to fit the binding isotherm data with non-linear regression to determine the change in enthalpy (ΔH) of the interactions. The standard deviations were obtained by two to three experimental repetitions.

Dynamic light scattering (DLS)

In order to form complexes of PF4 with antibodies, PF4 (20 µg/ml final) was incubated with aPF4/P-Abs (10 µg/ml final) at RT for 30 min before measurements. DLS-fixed scattering angle Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, UK) and disposal cuvettes were used. The Z-average size, i.e. the hydrodynamic diameter, of PF4, antibodies or their complexes was measured in PBS at 25 ºC and light scattering was detected at 173º using 10 repeating measurements. Data analysis was performed using the Zetasizer software, Version 7.11 (Malvern Instruments Ltd., Malvern, UK). DLS was also used to determine the zeta potential of the different anti-PF4/P Abs. Antibodies were diluted in water for zeta potential measurements which were recorded in a folded capillary zeta cell at 25°C with 6 repetitions.

Docking analysis of anti-PF4/P complexes

1E12, 1C12, and 2E1 cDNAs were sequenced by using the Sanger method, and a model of the 3D structure of these antibodies was then built by using MODELLER [22]. The structures used as templates for homology modeling were PDB:5MYO for the variable heavy (VH) chain and constant domains and PDB:4GMS for the variable light (VL) chain. The resulting 3D model was docked on PF4 (PDB: 1F9Q**)** [23], by using the method MAbTope [22,24] optimized for antibody/antigen complexes. Structures were imaged by using the PyMOL Graphics

System (v1.8). The prediction of the epitopes was based on the 30 best docking poses. Residues that were present at the interface in more than 20 poses had a very high probability to belong to the interface, those present in 15 to 19 poses, 10 to 14 poses and 5 to 9 poses had high, medium and low probability, respectively, to be part of the interface.

Ethics

The use of human sera obtained from healthy volunteers and patients with HIT including the informed consent procedure was approved by the ethics board at the University Medicine Greifswald. All volunteers gave informed consent; all studies were performed according to the declaration of Helsinki.

RESULTS

Antibody binding to PF4/H complexes and platelet activation properties

The binding to PF4/H complexes of all moAbs, KKO, 5B9, 1C12, 1E12, and 2E1 was dose-dependent as evidenced in ELISA. With 1C12, 1E12, and 2E1, OD values were high, almost maximal at a low concentration of antibodies (0.5 µg/mL), and their reactivity with PF4/H complexes was very similar to that of KKO. In contrast, OD was lower with 5B9, with a maximum value measured with 5 µg/mL, suggesting a lower affinity of 5B9 for PF4/H complexes (Fig. 1A).

Human antibodies, either non-platelet activating (group-1; non-pathogenic), or from typical HIT (group-2), or auto-immune HIT (group-3) patients, also displayed dose-dependent binding to PF4/H complexes by ELISA (Fig. 1B). The highest OD_{450nm} values were obtained with sera containing group-3 Abs, followed by group-2, and group-1 Abs. Binding patterns in

ELISA of human group-3 Abs resembled the pattern found with 1C12, 1E12, 2E1, and KKO while group-2 Abs exhibited a binding pattern similar to the ones found with 5B9.

In the presence of low concentrations of unfractionated heparin (UFH), KKO and 5B9 induced strong platelet activation (Fig. 1C) while no activation was observed in the absence of heparin. This platelet activation pattern is identical to that obtained with typical HIT Abs of group-2 (Fig. 1D). In contrast, 1C12, 1E12 and 2E1 activated platelets without addition of heparin (Fig. 1C), similarly to group-3 Abs from auto-immune HIT patients (Fig. 1D). High concentrations of heparin inhibited platelet activation induced by all monoclonal and human Abs tested.

Antibody binding strength and kinetics of interaction with PF4/H complexes

The binding strength and kinetics of interaction of moAbs and human Abs with PF4/H complexes were analyzed by single-molecule force spectroscopy (SMFS) (Fig. 2). Single IgG molecules of each antibody type were coated to a distinct AFM-cantilever tip and brought into contact with the surface coated with PF4/H complexes for measuring their binding strength/force **F** (Fig. 2A, B). Representative histograms of binding strength **F** measured with the moAbs (Fig. 2C) and human aPF4/P Abs (Fig. 2D) allowed the determination of mean interaction forces. Only some non-specific interactions were recorded with controls, including mouse IgG2b (mIgG2b) and human IgG (hIgG) purified from healthy donors. When moAbs were tested, the interaction forces measured were lower with KKO (42.0 ± 0.4 pN), than with 5B9 (67.9 ± 1.8 pN), while the highest values were obtained with 1C12 (77.7 ± 0.7 pN) and 1E12 (76.6 ± 0.9 pN). Notably, 2E1 exhibited a different reactivity pattern with PF4/H, i.e. yielding two different groups of interaction force values, at 64.1 ± 1.8 pN and 139.5 ± 2.0 pN, respectively. These data with moAbs were compared to those obtained with the three different groups of human Abs (Fig. 2D) [6]. The interactions forces measured with different sera were

relatively weak with group-1 Abs (46.3 ± 8.1 pN), stronger with group-2 Abs (60.6 ± 15.4 pN), and the highest values were recorded with group-3 Abs (72.4 ± 26.2 pN). These results are consistent with our previous findings [6].

To further understand the binding kinetics of reactions, we determined thermal off-rates (k_{off}) of the interactions between Abs and PF4/H complexes. For this, binding forces were recorded at velocities ranging from 10 to 4,000 nm/s for all antibody types. By analyzing binding forces at different loading rates (n = 3 repetitions) and applying the Bell-Evans model [20,21], we found different thermal off-rates (k_{off}) among antibody types (Fig. 2F). As an example, data for 5B9 are shown in Fig. 2E. For other Abs, rupture forces at different loading rates are given in supplementary material (Fig. S1). No k_{off} value as high as that of group-1 human Abs was obtained with any of the moAbs tested. The k_{off} values measured with KKO or 1C12 were comparable to that of group-2 Abs, and slightly higher values were recorded for 5B9 and 2E1. The lowest k_{off} (indicating the most stable binding) was obtained for 1E12, and group-3 Abs.

Antibody binding to PF4 on a solid phase and in solution

The binding properties of the five moAbs to PF4 immobilized on a solid phase were also assessed by SMFS. Each antibody was linked to an AFM tip while PF4 molecules were immobilized on the substrate, and interaction forces were compared to those obtained with the three groups of human aPF4/P Abs. As expected, the two moAbs, 5B9 and KKO, exhibited non-significant interactions with PF4 alone, comparable to control murine IgG2b. In contrast, 2E1, 1E12, and 1C12 clearly bound to PF4 (Fig. 3A), but to a lower extent than human group-3 Abs (Fig. 3B).

To evaluate the interactions of Abs with PF4 in solution, isothermal titration calorimetry experiments were performed. Very low heat release (ΔH) was measured when control IgG2b, KKO or 5B9 interacted with PF4 alone, (Fig. 3C, D, E), and values obtained were similar

when group-2 Abs were tested (Fig. 3J). In contrast, the heat released from the interaction of either 1E12 or 1C12 (Fig.3 F-H) with PF4 was higher and similar to the one obtained with human group 3 Abs (Fig. 3L). On the other hand, a different pattern was recorded with 2E1, as it released much lower heat than 1E12 and 1C12 when interacting with PF4 (Fig. 3 M, F, G, H). These data suggested that 1C12, 1E12 (and to a lesser extent 2E1), as well as human group-3 Abs, can cluster PF4 molecules and that this capacity is absent or very low for 5B9, KKO and group-2 Abs.

By dynamic light scattering (DLS) experiments, we also assessed the size of complexes formed in solution between PF4 and moAbs (Fig. 4A) or human Abs (Fig. 4B). As expected, this size was relatively high and similar when 1E12, 1C12, 2E1 or human group-3Abs were tested. On the other hand, KKO, 5B9 and group 2 Abs produced much smaller complexes with PF4 (Fig. 4).

Charge-related interactions of monoclonal anti-PF4(/P) Abs

We further evaluated by ELISA the binding of each monoclonal antibody to PF4 alone, to protamine alone, and to heparin immobilized by protamine, in the presence or absence of PF4. As expected, high OD values were measured with KKO, 5B9, 1C12, 1E12 and 2E1 in the presence of PF4/heparin complexes, and only 1C12, 1E12 and 2E1 interacted with PF4 alone (Fig.5A). But, surprisingly, significant binding of 2E1 to heparin immobilized by protamine in the absence of PF4 was also measured, without any interaction with protamine alone. This supported that this antibody, apart from binding to PF4, also interacted with heparin alone. Next, we determined the zeta potential of all antibodies, which was either neutral (5B9 and human group-3 abs), slightly negatively charged (1E12, 1C12 and human group-2 abs), or

strongly negatively charged (KKO and human group-1 abs). A positive zeta potential was only found for 2E1 (Fig. 5B).

Prediction of antibody binding sites on PF4 by a docking model

The binding sites of 1C12, 1E12, and 2E1 to PF4 were predicted using the MAbTope method, based on the VH and VL sequences of each monoclonal antibody and the crystal structure of PF4 tetramer, and our data were compared to those obtained previously with 5B9 [10], and by crystallography with KKO [23]. According to our model, 1C12, 1E12, and 2E1 appear to bind to monomers B, C, and D (Fig. 6) and to interact with 23, 31 and 21 AA residues, respectively. It is noteworthy that 15 amino acid residues (6, 4 and 5 in monomers B, C, and D, respectively) are likely involved in the binding of the three aPF4(/P) moAbs, including 8, 5, 8 with a very high or high probability for 1C12, 1E12, and 2E1, respectively. Six additional residues are also potentially contributing to the binding site, each interacting with variable combinations of 2 antibodies. Eleven other amino acids have also been identified to interact exclusively with 1E12, and only 2 or 5 with 2E1 and 1C12, respectively.

DISCUSSION

In this study, we analyzed all currently available monoclonal Abs, which bind to PF4(/P) and activate platelets (5B9; KKO; 1C12; 1E12; and 2E1), and compared their characteristics with those of human Abs affinity-purified from sera of patients with typical or autoimmune heparin-induced thrombocytopenia.

The three new monoclonal IgG Abs with a human Fc-fragment, 1C12, 1E12, and 2E1, clearly differ in terms of specificity and functional activity from the monoclonal anti-PF4/H IgG Abs,

KKO and 5B9 [8,10]. Indeed, 5B9 and KKO exhibit similar properties as typical heparin-dependent, platelet-activating human HIT Abs (group-2), whereas 1C12, 1E12, and 2E1 behave similarly to human Abs of patients with "auto-immune" HIT (group-3).

1C12, 1E12, 2E1, and group-3 Abs displayed a very high affinity for PF4/P complexes in comparison with 5B9, as suggested by the ten times lower concentrations necessary for reaching maximum OD values in ELISA. These Abs, therefore, resembled human group-3 Abs, which OD values were systematically higher compared to the ones obtained with human group-2 Abs. The higher affinity of 1C12, 1E12, 2E1 was also confirmed by SMFS measuring very high interaction forces of these Abs with PF4/P complexes, similarly to those recorded with human group-3 Abs. In contrast, interaction forces of 5B9 and KKO with PF4/P complexes were lower and close to those measured with human group-2 Abs. The high OD values obtained in ELISA with KKO, despite the lower interaction forces with PF4/P complexes, compared to 5B9, can be explained by a more stable binding of this monoclonal Ab with a lower thermal-off rate. Our data thus illustrate that ELISA results are always resulting from a variable composite of antibody affinity and avidity.

1C12, 1E12, and 2E1, similarly to human group-3 Abs associated with autoimmune HIT, also bound to PF4 alone as shown by ELISA, SMFS, ITC, and DLS. In contrast, 5B9, KKO, and group-2 Abs bound only to PF4/heparin complexes. Interestingly, Sachais et al. had also found that KKO was able to induce the oligomerization of PF4 [25]. However, in our hands only a small increase in aggregate size (measured by DLS) was evidenced when KKO, 5B9 and group 2 Abs were incubated with PF4, and no major changes in energy release were measured. This was contrary to what we observed with 1C12, 1E12, 2E1, or human group 3 Abs, which formed larger aggregates in the presence of PF4 alone than KKO, 5B9, and human group-2 Abs. This raises the interesting possibility that 1C12, 1E12, and 2E1 may facilitate the formation of ultra-large complexes (ULC) of PF4 on the platelet surface, which

is highly critical in the pathogenesis of HIT [26]. This hypothesis is supported by the ability of 1C12, 1E12, and 2E1 to strongly activate platelets without heparin, to a similar extent than group-3 Abs purified from "auto-immune" HIT patients. Moreover, platelet activation was fully inhibited in the presence of high concentrations of heparin, confirming that dissociation of PF4 complexes from the platelet surface fully abrogated platelet activation induced by 1C12, 1E12, and 2E1. It has recently been proposed that autoimmune HIT Abs can cluster PF4 and change its conformation, which allows binding of heparin-dependent Abs [6,27]. Our findings are consistent with this hypothesis, but it remains unclear, whether the same effects are caused by all three monoclonal Abs tested. This requires further studies, especially as we now know that the characteristics of different autoimmune HIT like monoclonal Abs may differ. Although the binding sites for 1C12, 1E12, and 2E1 on PF4 seem to be very close to each other, as predicted by our docking model, some differences were evidenced in SMFS and ITC experiments. The lowest k_{off} value in SMFS was measured with 1E12, indicating this antibody binds to PF4/P complexes with highest stability. These features were also the closest to those of human auto-immune HIT group-3 Abs [6]. Moreover, 1E12 induced the highest thermal energy release in ITC suggesting that this antibody can elicit major conformational changes of PF4. On the other hand, intriguing data were obtained with 2E1, with a bimodal distribution of its interaction forces in SMFS, suggesting variable antibody binding modalities with PF4/P complexes. In addition, the high thermal-off rate (k_{off}) combined with relatively low thermal energy release (compared to 1E12 or 1C12) also supported unstable 2E1 binding and reduced ability of this Ab to induce conformational changes of PF4. This led us to hypothesize that 2E1 could also interact with PF4/P complexes by charge-related binding. Binding of 2E1 to heparin was confirmed by ELISA experiments, in which 2E1 was shown to also bind to heparin alone.

As an antibody cannot bind with one antigen recognition site to two structurally different epitopes as exposed on heparin versus PF4, the most likely explanation for this apparent "double binding" was the implication of charge-related interactions. We, therefore, assessed the Zeta potential of these Abs in which 2E1 showed the strongest positive charge of all Abs tested. Thus, we propose that 2E1 involves two binding modalities to PF4/heparin complexes. One is binding to heparin alone, as shown by ELISA in the absence of PF4, likely caused by electrostatic interactions between a positively charged region of 2E1 (not yet identified) and the negative charge of heparin. The other one is heparin-independent binding of 2E1 to PF4, as shown by ELISA and SMFS, which likely involves a binding site similar to those of 1C12 and 1E12, according to our docking model. On the other hand, some residues, Q9, P34, and P37, which are critical for the antigenicity of PF4 in humans and for the interaction of KKO and 5B9 with PF4/P complexes [10,23,28,29], are also likely participating in the binding of 1C12, 1E12, and 2E1.

The characteristics of our three new monoclonal antibodies suggest that the reactivity patterns of antibodies associated with autoimmune HIT can be attributed to at least two different mechanisms. The first one is strong antibody binding to PF4 inducing a conformational change with consecutive binding of other Abs, as shown with human HIT group-3 Abs. The other one is weak antibody binding to PF4 via the antigen-binding site with additional charge-related interaction with heparin or other polyanions on the platelet surface like chondroitin sulfate or polyphosphates [30]. These Abs cannot be differentiated in functional assays since they both strongly activate platelets independent of heparin via FcγRIIA, which is inhibited by high concentrations of heparin. If such Abs may also occur in humans it is likely that they will interact with heparan sulfate on endothelial cells, which might be highly relevant for pathological consequences.

For many years, we have known that the specificity and functional properties of IgG Abs to PF4 can be variable from one patient with HIT to another. In addition, several IgG Abs with different characteristics may coexist in the same individual, as first showed several years ago [31], and recently confirmed [6].

In conclusion, the well-characterized different monoclonal aPF4 IgG Abs, which are available today, are potentially useful tools to further investigate the pathophysiology of HIT, including the hypothesis of cooperation among different IgG Abs, which may impact the extent of cell activation and the occurrence of thrombotic complications.

Author's Contributions

C. V and T. H. N performed and designed the research, analyzed data and wrote the paper. J. R, A. G and Y. G designed the research, analyzed data, and wrote the paper. C. P analyzed data and wrote the paper. N. C, A. P and N. N performed research and analyzed data. All authors reviewed and approved the final version of the manuscript.

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Conflict of Interest

The authors declare they have no conflicts of interest.

Supplementary material

Additional material may be found in the online version of this article:

Figure S1: Binding kinetics of antibodies to PF4/H complexes.

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LEGENDS OF FIGURES

Figure 1: Binding to PF4/H complexes and platelet activation properties of murine monoclonal and purified human anti-PF4/P IgG antibodies

Mean OD values ± SEM obtained after incubation with coated PF4/H complexes of variable concentrations of (**A**) monoclonal aPF4/(P) antibodies KKO, 5B9, 1E12, 1C12, 2E1 (n=3 experiments) and (**B)** human aPF4/P Abs (groups 1-3) purified from five human sera for each group. Absorbances were measured by different enzyme immunoassays and photometers and OD values are therefore not directly comparable. (**C**) Serotonin release assay performed with 2E1, 1E12 and 1C12 in comparison with 5B9 and KKO, with and without unfractionated heparin. Data are mean (%) +/- SEM of results obtained with 3 different platelet donors for each monoclonal antibody. (**D**) HIPA performed with affinity-purified PF4-specific IgG from patients of groups 1-3. The low molecular weight heparin reviparin was used in the HIPA test at low concentration (0.2 aFXaU); n = 5 donors tested for each antibody group. Data are given as lag time until platelet activation (+/- SEM); as shorter the lag time as stronger the reactivity.

Figure 2: Binding strength of PF4(/P) moAbs and human Abs to PF4/H complexes

(**A**) A single antibody was attached covalently on an AFM-tip via PEG linkers, and PF4/H complexes were immobilized on the substrate for measurement of their interaction force **F**.
(**B**) Example of force-distance curves showing force **F** obtained with 5B9 and 2E1.
(**C, D**) Rupture forces and corresponding standard errors determined by a Gaussian fit (solid curves) with (**C**) moAbs KKO, 5B9, 2E1, 1E12, 1C12 and mIgG2b, or (**D**) human Abs of groups 1-3 and human control IgG (hIgG).

(**E**) Representative rupture forces recorded for 5B9 at different loading rates allowed to determine thermal off-rate (k_{off}) of the interaction.

(F) Average k_{off} values and standard deviations obtained with all moabs and human Abs studied (n = 3 repetitions) are shown. *Note*: low k_{off} = stable binding. mIgG2b = mouse control IgG, hIgG = human control IgG.

Figure 3: Binding of monoclonal and human IgG Abs to PF4 in SMFS and ITC

(**A**) Binding strength of moAbs (left panel) and (**B**) polyclonal human Abs (right panel) to PF4 alone measured by SMFS (inset, **A**). (**C-K**) Representative patterns of integrated heat released by interactions of different Abs with PF4 measured by ITC. (**M**) Summary data of mean +/- SD from n = 2 ITC repetitions.

Figure 4: Oligomerization of PF4 induced by monoclonal and human Abs assessed by DLS

Size of PF4-IgG complexes with (**A**) MoAbs and **(B)** human Abs. Data are mean diameter (nm) +/- SD from 3 repetitions.

Figure 5: Surface charge of monoclonal Abs and impact on binding specificity

(**A**) Zeta potential of human and monoclonal anti-PF4(/P) IgG Abs measured in DLS (n=3).

(**B**) Binding of anti-PF4(/P) moabs to protamine, heparin and PF4 complexes. OD values obtained after incubation of 5B9, KKO (10 µg/mL), 1C12, 1E12, and 2E1 (0.5 µg/mL) in wells coated with protamine (white), protamine/heparin (black), protamine/heparin/PF4.

Figure 6: Epitope mapping of antibody-PF4 interactions of 2E1, 1C12, 1E12 compared to 5B9 and KKO

Residues of PF4 monomers (A) are colored according to their probability of contributing to the epitopes recognized by Moabs, as given by MAbTope. Colors for 1C12, 2E1, 1E12 are: dark red (very high probability), red (high probability), orange (medium probability), and yellow (low probability). Colors for 5B9 are dark blue (very high probability), blue (high probability), cyan (medium probability), and light cyan (low probability). Residues of the KKO epitope are shown in green**. (B)** Surface and cartoon views of PF4 tetramer are shown in shades of grey (white: chain A, light grey: chain B, dark grey: chain C, black: chain D).