Heparin-induced thrombocytopenia: new evidence for the dynamic binding of purified anti-PF4–heparin antibodies to platelets and the resultant platelet activation

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Immune heparin-induced thrombocytopenia (HIT) is a potentially serious complication of heparin therapy. Patient plasma usually contains antibodies that aggregate platelets in the presence of heparin. In addition, antibodies directed against complexes of platelet factor 4 (PF4) and heparin are associated with this disease. However, HIT plasma that aggregates platelets does not always contain anti-PF4–heparin antibodies and vice versa. Understanding how HIT antibodies interact with platelets and whether the anti-PF4–heparin antibodies bind platelets and cause aggregation is important for understanding the pathophysiology of HIT. Unfortunately, conflicting models have been proposed to describe the interaction of HIT antibodies with platelets, and none of the previous studies directly investigated the binding of affinity-purified anti-PF4–heparin IgG to platelets.

Visentin et al. report that, with flow cytometry, IgG from some patients with HIT bound to resting, washed platelets. Blocking the platelet Fc receptor (FcγRII) with the monoclonal antibody IV.3 abolished antibody binding, which implied that only the Fc portion of HIT IgG interacts with platelets. Visentin et al. and others contend that HIT IgG forms an immune complex with circulating PF4–heparin and that the complex binds to the platelet FcγRII through the Fc portion of the IgG and causes platelet activation.

This model is disputed by Horne and Alkins, who investigated the binding of iodinated total IgG from patients with HIT to washed platelets. Antibody binding was reliably observed only with thrombin-activated platelets and was dependent on the addition of approximately equimolar concentrations of PF4 and heparin. In contrast to the findings of Visentin et al., they observed that Fc receptors were not important for the binding of HIT antibodies. Importantly, platelet aggregation was induced by the affinity-purified HIT IgG from HIT plasma. This IgG provides much greater sensitivity and is a more clearly defined antibody than the total IgG or plasma used by others. Importantly, platelet aggregation was induced by the affinity-purified HIT IgG, and antibody binding was studied in a plasma milieu with platelets exposed to shear forces. Our data also help to resolve the role played by the platelet FcγRII in HIT, which previously had only been investigated in artificial experiments.

Introduction

Immune heparin-induced thrombocytopenia (HIT) is associated with antibodies directed against a complex of platelet factor 4 (PF4) and heparin. We were able to affinity purify anti-PF4–heparin IgG (HIT IgG) from the plasma of 2 patients with HIT. Under conditions that were more physiological and sensitive than those in previous studies, we observed that this HIT IgG caused platelet aggregation on the addition of heparin. Platelets activated with HIT IgG increased their release and surface expression of PF4. We quantitated, for the first time, the binding of affinity-purified HIT iodine 125–IgG to platelets as they activated in a plasma milieu. Binding of the HIT IgG was dependent on heparin and required some degree of platelet activation. Blocking the platelet FcγRII with the monoclonal antibody IV.3 did not prevent HIT IgG binding to activated platelets. We concluded that anti-PF4–heparin IgG is the component in these HIT plasmas that induces platelet aggregation. The Fab region of HIT IgG binds to PF4–heparin on the surface of activated platelets. We propose that only then does the Fc portion of the bound IgG further activate the same or adjacent platelets through the Fc receptor. Our data support a dynamic model of platelet activation in which released PF4 enhances further antibody binding and more release.
Materials and methods

Purification of HIT IgG

Plasma was collected with informed consent from healthy individuals and from 2 patients with HIT (patient 1 and patient 2). The affinity purification of HIT anti-PF4–heparin IgG (HIT IgG) and a clinical summary of these patients has been previously described. Briefly, patients 1 and 2 were 72- and 67-year-old women. Thrombocytopenia developed in each of them after approximately 1 week of heparin therapy, and findings on 12C-serotonin release and platelet aggregation assays were positive. Other causes of thrombocytopenia were clinically excluded.

HIT plasma was passed through a heparin-PF4–agarose column, and bound antibodies were eluted with 0.13 to 1.5 mol/L NaCl gradient. IgG was further purified on a protein G Sepharose column. Normal IgG, which did not bind PF4–heparin, was purified from the pooled plasma of 3 healthy persons by affinity for protein G Sepharose. The molecular weights of the HIT and normal IgG were characterized under native conditions by size exclusion high-performance liquid chromatography using a Zorbax G-450 column (Hewlett Packard, Palo Alto, CA) and an aqueous solvent of 0.2 mol/L NaCl, 0.1 mol/L phosphate, pH 7.0, running at 1 mL/minute. The IgG was predialyzed against the running buffer and was detected at 210 nm. The IgG eluted as a single peak with an average molecular weight of 157 kDa when compared with gel filtration molecular weight standards (Bio-Rad Laboratories, Hercules, CA). No larger IgG aggregates were present. IgG was iodinated with Na-125I using iodosgen (Pierce; Rockford, IL).

IgG binding during platelet aggregation

The methods for platelet preparation and platelet aggregometry are based on those previously described. Platelet-rich plasma (PRP) was prepared by centrifuging citrated blood at 170g for 15 minutes. Plasma was obtained by centrifuging the remaining blood at 1600g for 15 minutes. The concentration of platelets was determined with an automated blood cell counter (Sysmex NE-8000, Kobe, Japan). Platelet aggregation was performed in silicon-coated glass tubes at 37°C by monitoring the increase in optical transmittance of the platelet suspension with a ChronoLog (Chrono-Log, Havertown, PA) aggregometer. Aggregation was recorded once a second on a personal computer with an 8-bit analog-to-digital converter (Pocket Sampler; Jaycar Electronics, Sydney, Australia).

The aggregation reaction involved stirring PRP (300 × 10⁶ platelets/mL) with HIT IgG (12 µg/mL = 80 nmol/L, 125I-IgG tracer (<0.07 µg/mL, ~13 kBq/mL), in 650 µL plasma. Aggregation was induced by the addition of heparin (0.5 µM/mL) or collagen (4µg/mL). The aggregation reaction was sampled by removing, in duplicate, 80 µL platelets with a -counter. The amount of bound 125I-IgG remained low throughout, indicating minimal entrapment of IgG due to the Fab region of HIT IgG, probably binding to PF4–heparin.

PF4 assay

A competition radioimmunoassay was developed to measure the PF4 concentration in plasma derived from activated and inactivated platelets (see above). Microtiter wells that could be individually separated (MaxiSorp–BreakApart; Nunc, Roskilde, Denmark) were coated with affinity-purified sheep anti-PF4 IgG and blocked with BSA. Dilutions of test plasma and purified PF4 standards (0.2 µg/mL) were prepared in a solution containing 1% BSA, 0.38% trisodium citrate, 10% ETP, and PBS–Tween. The well plasma contained 0.5 µM/mL heparin, the same heparin concentration was maintained throughout dilution of the test and standard PF4 solutions. Standard or unknown PF4 solution (110 µL) was equilibrated for 30 minutes with an equal volume of 125I-PF4 solution (~0.2 µg/mL, 4 KBq/mL) containing polycarbonate (50 µg/mL). One hundred microliters of PF4 mixture was incubated, in duplicate, for 15 minutes in the wells coated with anti-PF4 IgG. Wells were washed 3 times with PBS–Tween and then broken apart, and radioactivity was measured in a γ-counter. The PF4 concentration was determined by comparing counts with the appropriate standard curve, depending on the presence or absence of 0.5 µM/mL heparin. The standard curve with heparin was shifted by 0.25 µg/mL to the right of the curve without heparin. The most sensitive region of the PF4 standard curve was 0.05 to 1 µg/mL PF4, and samples were diluted to fall within this range. Polybrene was required in this assay to prevent heparin from abolishing the binding between PF4 and antibody.

Results

HIT IgG binding during platelet aggregation

We investigated the binding of affinity-purified HIT 125I-IgG to platelets at various time points (A, B, C, and D) during platelet aggregation induced by the antibody and heparin (Figure 1). Before heparin was added to the suspension of PRP and HIT IgG (time point A), the platelets remained non-aggregated, and negligible binding of HIT IgG was detected. The addition of heparin triggered platelet aggregation. The beginning of aggregation (time point B) was associated with low but detectable binding of HIT IgG. At time point C, when platelets were approximately 50% aggregated, there was approximately half-maximal HIT IgG binding. The greatest IgG binding was observed once platelets were fully aggregated (time point D). The nonspecific binding of normal 125I-IgG remained low throughout, indicating minimal entrapment of IgG during aggregation. These data suggest that some degree of platelet activation is required for HIT IgG binding to platelets.

Role of platelet activation and platelet FcγRII

The role of the FcγRII in the binding of HIT IgG to platelets was investigated by blocking the FcγRII with the monoclonal antibody IV3. As expected, the presence of IV3 prevented HIT IgG and heparin from inducing platelet aggregation, and no binding of HIT 125I-IgG to these platelets was detected. However, after collagen was added to activate the platelets, the HIT 125I-IgG did bind (Figure 2). Thus, blocking the FcγRII did not prevent binding of HIT IgG if the platelets could be activated by another pathway. This indicates that IgG binding to activated platelets must have occurred by the Fab region of HIT IgG, probably binding to PF4–heparin.
induced aggregation was complete. Samples were centrifuged through a 17% sucrose cushion to separate platelets from unbound IgG. Specific binding of patient 1 (●) and patient 2 (●) HIT IgG was measured by using HIT 125I-IgG tracer, whereas nonspecific binding (open symbols) was determined with normal 125I-IgG instead. The gray trace shows a typical aggregation profile that progresses from unaggregated to fully aggregated on an arbitrary scale. IgG bound is the mean ± SE of 3 to 8 experiments.

**Specificity of affinity-purified HIT IgG**

To confirm that the HIT IgG indeed recognized PF4, we blocked the binding of HIT IgG to activated platelets by preincubating the platelets with affinity-purified sheep anti-PF4 IgG. The binding of HIT 125I-IgG tracer (without unlabeled HIT IgG) was increasingly inhibited with higher concentrations of anti-PF4 (Figure 4). At 48 µg/mL anti-PF4, the binding of HIT IgG from patients 1 and 2 was reduced to one quarter and one eighth, respectively, of the binding without anti-PF4.

**Release of PF4 from platelets**

To show that PF4 is released during platelet activation, we quantitated the PF4 released from platelets, anticoagulated with citrate, contained 0.19 µg/mL PF4. When platelets were aggregated by the addition of either HIT
IgG plus heparin, collagen alone, or collagen plus heparin, the plasma contained approximately 5 µg/mL PF4. Treatment of platelets with heparin alone released a lesser amount of PF4, and the resultant plasma contained 0.82 µg/mL PF4. The released PF4 may be absorbed onto the platelet surface, resulting in elevated surface expression of the protein. PF4 release by HIT IgG without heparin was not investigated because IgG alone does not trigger platelet activation.

Increased expression of PF4 on the platelet surface after activation

The relative surface expression of PF4 on activated and nonactivated platelets was measured by the binding of sheep anti-PF4 125I-IgG (Figure 6). Unstimulated citrated platelets displayed little specific binding of anti-PF4 antibodies. Aggregation of platelets with either HIT IgG plus heparin, collagen alone, or collagen plus heparin resulted in a marked increase in PF4 expression. Platelets treated with heparin alone had only a modest increase in surface PF4 compared with unstimulated platelets.

These data support the notion that the surfaces of activated platelets are covered in PF4 that binds heparin and HIT IgG. The bound IgG activates other platelets through FcγRII, which initiates a chain reaction that leads to a rapid increase in platelet aggregation and HIT IgG binding.

Role of heparin

Figure 7 demonstrates that heparin is required to facilitate the binding of HIT IgG to aggregated platelets. Platelets aggregated with collagen alone did not bind HIT IgG. Binding only occurred when heparin was included with the collagen. This supports the concept that PF4 must form a complex with heparin before it is recognized by HIT IgG.

The amount of IgG bound to platelets was double (Figure 7) the amount when FcγRII was blocked by IV.3 (Figure 2). This probably reflects differences in the degree of platelet activation between the 2 figures and not a component of Fc binding. In Figure 7 platelets were activated by both collagen and HIT IgG and so probably expressed more PF4–heparin antigen than did Figure 2, in which activation by HIT IgG was prevented by IV.3. Support for this view comes from our observation that platelets activated by HIT IgG plus heparin bound more anti-PF4 IgG than those activated by collagen plus heparin (Figure 6).

Discussion

In this study we demonstrated that HIT IgG, affinity purified on heparin-PF4–agarose, induces heparin-dependent platelet aggregation (Figure 1). This finding extends that of Greinacher et al., who showed that HIT antibodies, isolated by affinity for endothelial cells, could bind PF4–heparin on enzyme-linked
immunosorbent assay and activate platelets. However, our data contribute the strongest evidence that the anti-PF4–heparin antibody is the heparin-dependent platelet-aggregating factor, known to be present in the plasma of patients with HIT, and they imply that the anti-PF4–heparin antibody may cause in vivo platelet activation and, consequently, thromboembolism. However, this mechanism may not necessarily reflect the pathophysiology in all patients with HIT.

We provide, for the first time, fundamental data on the binding of affinity-purified HIT anti-PF4–heparin IgG to platelets during platelet aggregation. We used concentrations of platelets,21 heparin,22,23 and specific anti-PF4–heparin IgG17 that were achievable in vivo. Significantly, no exogenous PF4 was added, and PF4 released from the platelets was available to modulate further antibody binding to the platelets. Our findings are particularly significant because the platelet aggregation was induced by the HIT IgG itself rather than by thrombin. The use of affinity-purified antibody provided high sensitivity.

Figure 1 indicates that before the addition of heparin, there was little or no binding of HIT IgG to platelets. We only detected that HIT IgG bound to platelets when heparin was added to the platelets and aggregation began. The HIT IgG binding then increased sharply until platelets were fully aggregated. There is clearly a close correlation between the degree of aggregation and the amount of HIT IgG bound. This confirms that platelet aggregation by HIT IgG is a dynamic process by which activation promotes further antibody binding. We speculate that a very low level of HIT IgG binding to the platelets occurred before aggregation.

We confirmed that PF4 is strongly implicated in forming the platelet antigen recognized by HIT IgG because the binding of HIT IgG to activated platelets was inhibited by the preincubation of platelets with sheep anti-PF4 IgG (Figure 4). Furthermore, the activation of platelets by HIT IgG plus heparin, or by collagen, triggered a substantial increased in the expression of PF4 on the platelet membrane (Figure 6). Of note, incubating platelets with heparin alone was sufficient to elevate modestly the surface expression of PF4.

Many reports demonstrate that HIT antibodies optimally bind to PF4–heparin in a narrow range of PF4:heparin ratios. In our hands, this ratio corresponded to at least 20 µg PF4/U heparin, regardless of whether the PF4–heparin was immobilized on enzyme-linked immunosorbent assay wells or was free in solution. We have demonstrated that the ratio of PF4:heparin is perhaps not as important in a more physiological and sensitive system. We added 0.5 U/mL heparin to platelet-rich plasma, but the PF4 was derived solely from that already present in the plasma or the platelets, or both. The plasma PF4 concentration increased from <0.19 µg/mL in citrated platelet-rich plasma to ≈5.6 µg/mL when platelets were fully aggregated by HIT IgG (Figure 5). Consequently, heparin was always in excess, but the increase in PF4 concentration would have moved the PF4:heparin ratio toward the optimum. It is likely that this contributed to the increased binding of HIT IgG during platelet aggregation.

Previously, we and others5,10,17,20 observed that HIT IgG binds weakly to PF4 alone, immobilized into microtiter wells in the absence of heparin. Therefore, we investigated the importance of heparin in the binding of HIT IgG to platelet-surface PF4. We observed that binding of anti-PF4–heparin IgG to collagen-aggregated platelets only occurred in the presence of heparin (Figure 7), despite the fact that platelets aggregated by collagen alone expressed significant amounts of PF4 on their surfaces (Figure 6). We concluded that PF4 on the platelet surface may not be modified in the same way as PF4 adsorbed into microtiter wells. Instead heparin is required to facilitate HIT antibody binding, perhaps by inducing a conformational change within PF4.

Aster21 indicates that understanding the orientation of IgG on the platelet surface is important. If the Fab region of IgG can bind platelets, then anti-PF4–heparin antibodies of IgA or IgM classes may opsonize platelets for destruction in vivo, and this may explain why HIT can develop in patients with only IgA or IgM anti-PF4–heparin antibodies.25 Figure 2 indicates that, as expected,26,27 IV.3 prevented HIT IgG from aggregating platelets in the presence of heparin, and these nonaggregated platelets did not bind HIT 125I-IgG. Platelet aggregation was then induced by the addition of collagen, which acts through a mechanism independent of FcγRII. Once aggregated, the platelets bound HIT IgG despite the presence of IV.3. Similarly, F(ab')2 fragments of HIT IgG also bound to platelets aggregated by collagen and heparin (Figure 3). This implies that the binding of HIT IgG to platelets occurs when the Fab portion of IgG recognizes PF4–heparin already on the platelet surface. Consequently, HIT IgG binding is dependent on platelet activation—needed to increase PF4 expression on the platelet surface—and not on the availability of FcγRII. The effect of IV.3 is simply to prevent HIT IgG from activating platelets through the Fc receptor.

We present definitive evidence that anti-PF4–heparin IgG from patients with HIT can cause heparin-dependent platelet aggregation. For the first time, we also report the dynamic binding of HIT IgG to platelets as the IgG (plus heparin) induces platelet aggregation under conditions that are as close as possible to those found in vivo. Our data support a mechanism of platelet activation by HIT IgG that is summarized in Figure 8. Initially, on the addition of heparin, tiny amounts of PF4–heparin complexes form on the platelet surface. The Fab portion of HIT IgG binds to this antigen, and the Fab region of the bound HIT IgG cross-links FcγRII on the same or adjacent platelets. This triggers platelet activation and degranulation. The PF4 that is released binds more heparin and forms more antigen on platelets. Thus, positive feedback accelerates platelet activation until all the platelets are fully aggregated.

These data on the dynamic binding of HIT IgG to platelets in the presence of heparin provide further insights into the pathophysiology of heparin-induced thrombocytopenia and thrombosis.

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References