Dimeric FcγR ectodomains detect pathogenic anti-platelet factor 4–heparin antibodies in heparin-induced thrombocytopenia

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Introduction

Heparin-induced thrombocytopenia (HIT) occurs when antibodies form immune complexes (ICs) with platelet factor 4 (PF4) bound to heparin or glycosaminoglycans [1–3]. The pathogenic ICs bind to FcγRIIa, which is the only FcγR on platelets, triggering their activation and aggregation, leading to thrombosis. Binding to FcγRIIa on monocytes also causes both prothrombotic production...
of thrombin and tissue factor [4] and the clearance of platelets and thrombocytopenia [5]. Many patients treated with heparin develop antibodies against PF4–heparin, but the presence of antibody–PF4–heparin complexes does not necessarily result in clinical manifestations of thrombosis/thrombocytopenia. Antigen recognition-based methods (e.g. ELISA) detect anti-PF4–heparin antibodies, but fail to distinguish pathogenic from non-pathogenic antibodies. Thus, platelet functional assays, such as the serotonin release assay (SRA), are the most reliable for confirming HIT [2,6,7], but require access to appropriate donor platelets that are sensitive to activation, and are not easily replicated between many clinical laboratories.

The mAb KKO binds the PF4–heparin complex and activates human platelets in an FcγRIIa-dependent manner [8]; it causes HIT in a human FcγRIIa/PF4 transgenic mouse model [9,10]. A recent X-ray crystallography analysis showed the KKO mAb bound to a conformation-dependent epitope on heparin-related pentasaccharide (fondaparinux)-bound PF4 tetramers, promoting the formation of higher-order complexes [11,12]. In contrast, a non-pathogenic antibody bound an overlapping epitope, but only in the PF4 monomer. Plate-based ELISAs present a heterogeneous mixture of PF4 forms, and so do not distinguish innocuous antibodies from those forming complexes capable of activating FcγRs.

Pathological HIT antibodies engage FcγRIIa, and trigger platelet activation and clearance [3] and tissue factor production [4]. The pathology depends, in part, on an R131H polymorphism within FcγRIIa, which does not alter the expression levels of the receptor but does significantly alter the affinity of FcγRIIa for its ligand [13]. We recently described the use of dimeric recombinant soluble FcγRIIa (rsFcγRIIa) to determine the proximity of pairs of IgG antibodies in immune cell-activating ICs [14]. The binding of dimeric rsFcγRIIa in this assay is correlated with the capacity of IgG ICs to activate FcγR-dependent cellular responses [14,15]. In this study, we tested the capacity of this unique dimeric rsFcγRIIa to distinguish pathogenic antibodies, which recognize PF4–heparin complexes and are able to activate platelets, from clinically irrelevant, non-pathogenic antibodies.

### Materials and methods

Plasma samples were obtained from 27 medical and surgical inpatients based at a tertiary hospital, the Royal Adelaide Hospital, in Adelaide, Australia, in whom HIT was suspected. Local ethics committee approval was obtained prior to the commencement of the study. The collection of samples conformed to institutional guidelines. Both plasma from citrated blood and sera were prepared for analysis. For the purposes of this study, and to ensure that HIT cases reflected the integration of both clinical and laboratory criteria, a diagnosis of HIT was defined as a 4T score of ≥ 4 and a positive SRA result (> 20% at 0.1 U mL⁻¹ heparin, and suppression at 100 U mL⁻¹ heparin) [16]. Levels of PF4–heparin autoantibodies were analyzed with an IgG-specific solid-phase ELISA (GTI, Waukesha, WI, USA) [17] and with the HemosIL AcuStar HIT IgG-specific assay (Instrumentation Laboratory, Bedford, MA, USA) [18] under standardized laboratory conditions. High specificity with the HemosIL AcuStar HIT IgG-specific assay has been previously reported [17].

The production and use of dimeric rsFcγRIIa (His131 allele form) has been described previously [14]. To assess the ability of dimeric rsFcγRIIa to differentially bind pathogenic versus non-pathogenic HIT antibodies, patient plasma was used in a PF4–heparin IgG ELISA kit (Diagnostica Stago, Melbourne, Victoria, Australia) at a plasma dilution of 1 : 40, and with incubation and washing steps according to the manufacturers’ instructions. The bound anti-PF4–heparin antibodies were then reacted with dimeric rsFcγRIIa–biotin (0.2 μg mL⁻¹) in phosphate-buffered saline (PBS) diluent containing 1 mM EDTA, 0.05% (v/v) Tween-20 and 1% (w/v) bovine serum albumin for 1 h at 37 °C, and this was followed by five cycles of filling and aspirating wells with wash buffer (PBS, 0.05% v/v Tween-20). Bound receptor was then detected by the incubation with a 1 : 10 000 dilution of high-sensitivity horseradish peroxidase–streptavidin (Pierce, Rockford, IL, USA), for 1 h at 37 °C, washed 10 times, and incubated with TMB Single solution (Life Technologies, Mulgrave, Victoria, Australia). The reaction was terminated with an equal volume of 1 m HCl, and absorbance at 450 nm was determined. An in-house plasma sample from a patient with confirmed HIT (4T score of 8, an SRA release of 94.2% with suppression of serotonin release with high-dose heparin, and an anti-heparin–PF4 antibody OD of > 3.0 by IgG ELISA) was used as a standard to define a nominal 100% response, to which absorbance values were normalized.

Statistical analysis was performed with graphPad Prism version 6.05 (GraphPad Software, San Diego, CA, USA). The optimal OD cut-off for the dimeric rsFcγRIIa assay (normalized value = 3) was confirmed by receiver operating characteristic analysis (not shown). Cut-off values for the PF4–heparin IgG ELISA (OD = 0.4) and for the HemosIL AcuStar HIT IgG-specific assay (1 U mL⁻¹) were defined according to the manufacturers’ instructions. SRA positivity was defined as > 20% serotonin release with low-dose heparin at 0.1 U mL⁻¹, and < 20% release with a high heparin concentration of 100 U mL⁻¹.

### Results and discussion

A cohort of 27 patients with suspected HIT were evaluated, of whom 13 were considered to be HIT-positive because of a positive SRA finding and a 4Ts score of ≥ 4 [6]. Samples were also evaluated for anti-PF4–heparin antibodies with PF4–heparin IgG ELISA and the HemosIL AcuStar HIT IgG-specific assay. The ability of a
novel engineered dimeric rFcγRIIa (His131) to detect pathogenic anti-PF4–heparin antibodies was evaluated by comparison with these established assays.

The novel dimeric rFcγRIIa assay, AcuStar HIT IgG-specific assay and PF4–heparin IgG ELISA all showed significantly higher mean activity for HIT-positive samples than for HIT-negative samples (all assays, Mann–Whitney U-test P < 0.0001; Figure 1A). The performance of the assays in distinguishing HIT patients is summarized in Table 1. The dimeric rFcγRIIa assay correctly assigned 93% of the patients (25/27) and did not detect two HIT samples (i.e. two false negatives). This was similar to the performance of the AcuStar HIT IgG-specific assay, which correctly assigned 96%, with only one false negative. These two assays contrasted with the more sensitive PF4–heparin IgG ELISA, which had a lower specificity (71%), with four false positives from this cohort. Larger patient groups that include differing clinical groupings will be necessary to establish robustly the predictive value of this approach.

A comparison of the dimeric rFcγRIIa and AcuStar HIT IgG-specific assays for the HIT-positive samples showed that these two assays correlated more strongly with each other (Spearman’s correlation r = 0.845, P = 0.0005) than either did with the PF4–heparin IgG ELISA (ELISA versus AcuStar assay, r = 0.813, P = 0.0015; ELISA versus dimeric rFcγRIIa assay, r = 0.673, P = 0.015).

As the SRA is a definitive functional assay for the diagnosis of HIT, and the main limitation of the PF4–heparin IgG ELISA is that it gives false positives, we examined the samples that were positive with the PF4–heparin IgG ELISA (Figure 2A) and the relationship between their activities in the SRA and in the other assays (Figure 2B, C). Both the AcuStar HIT IgG-specific and dimeric rFcγRIIa assays appropriately assigned low, below cutoff, values to the SRA-negative samples, although some were very near the cutoff values of both assays. Furthermore, the AcuStar HIT IgG-specific and dimeric rFcγRIIa assays also correctly assigned as negative a sample (see filled blue star; Figure 2) that had ~50% release at both high and low heparin doses, and was weakly positive with the PF4–heparin IgG ELISA. The AcuStar HIT IgG-specific and dimeric rFcγRIIa assays, although correlating strongly (Figure 1B), showed some important differences (Figure 2B,C). One HIT patient plasma sample was weakly AcuStar HIT IgG-specific assay-positive (1.78 U mL−1; cut-off of 1.0 U mL−1) and dimeric rFcγRIIa assay-negative (0.4% response; cut-off

| Assay                | Positive test | Negative test | Specificity | Sensitivity |
|----------------------|---------------|---------------|-------------|-------------|
| Dimeric rFcγRIIa     | 11            | 0             | 1.00        | 0.85        |
| PF4–heparin IgG      | 13            | 4             | 0.71        | 1.00        |
| AcuStar HIT IgG      | 12            | 0             | 1.00        | 0.92        |
| AcuStar HIT IgG-specific | 12            | 0             | 1.00        | 0.92        |

PF4, platelet factor 4; rFcγRIIa, recombinant soluble FcγRIIa.

Fig. 1. Comparison of the novel dimeric recombinant soluble FcγRIIa (rsFcγRIIa) assay with existing serological assays for the detection of heparin-induced thrombocytopenia (HIT) antibodies. (A) Suspected HIT patients were segregated on the basis of a positive serotonin release assay (SRA) result and a 4Ts score of ≥ 4. The three dashed lines indicate the cut-off values for a positive result with each assay. These thresholds were 3% of the normalized signal for the dimeric rsFcγRIIa assay: 1 U mL−1 for the AcuStar HIT IgG-specific assay, and an OD of 0.4 (right y-axis) for the platelet factor 4 (PF4)–heparin IgG ELISA. Two sets of parentheses mark the false negatives from the assays: two from the dimeric rsFcγRIIa assay, and one from the AcuStar HIT IgG-specific assay. The third set of parentheses marks the four false positives from the PF4–heparin IgG ELISA. (B) The dimeric rsFcγRIIa and AcuStar HIT IgG-specific assays were correlated for the HIT-positive (i.e. 4T score of ≥ 4 and SRA-positive) samples. Cut-off values for the two assays are shown as dotted and dashed lines, respectively. HIT samples with low antibody levels are more sensitively identified by combining both the dimeric rsFcγRIIa and AcuStar HIT IgG-specific assays. When HIT positivity is defined as rsFcγRII a a positivity and/or AcuStar HIT IgG-specific assay positivity, sensitivity is improved over that with either assay used alone.
of 3%; open triangle, Figure 2). Another HIT patient was weakly positive with the AcuStar HIT IgG-specific assay (1.05 U mL\(^{-1}\)) but scored strongly in the dimeric rsFc\(\gamma\)RIIa assay (67% response; open square, Figure 2). Thus, defining samples as HIT-positive according to AcuStar HIT IgG-specific assay-positive and/or dimeric rsFc\(\gamma\)RIIa assay-positive criteria may be an approach for improving HIT prediction with either assay alone.

In HIT, pathogenic PF4–heparin IgG complexes engage and aggregate Fc\(\gamma\)RIIa on platelets [1,3,5], and possibly other Fc\(\gamma\)Rs on monocytes [4,19]. The size and stoichiometry of IgG ICs are major determinants of both the avidity of interaction with the low-affinity Fc\(\gamma\)Rs [20] and the capacity of anti-PF4–heparin antibodies to activate cells. In a large proportion of laboratories, the initial clinical suspicion of HIT is followed by the performance of standard HIT IgG ELISAs. However, the diagnosis of HIT and subsequent decisions regarding patient anticoagulation and management are made challenging by the prevalence of non-pathogenic anti-PF4–heparin antibodies that are unable to activate Fc\(\gamma\)Rs. Although the levels of anti-PF4–heparin antibodies as detected by HIT IgG ELISA provide useful information, this measurement alone does not provide sufficient specificity for the diagnosis of HIT [7,21].

Thus, a platelet functional HIT assay, such as platelet aggregation or the SRA, is crucial for diagnosis. The SRA, however, involves handling of radioactive isotopes and requires specialized personnel, specialized equipment and rapid access to functional ‘high-responder’ platelets that may not be routinely available. Use of the SRA is also limited to major reference laboratories. Refinements to improve the specificity of the HIT anti-PF4–heparin antibody ELISA have included raising the OD cut-off for positive results and the addition of a high-dose heparin confirmatory step [16,22]. However, there is still a need for improved serological HIT assays.

We investigated the potential of dimeric rsFc\(\gamma\)RIIa, a probe of the Fc\(\gamma\)R-mediated effector functionality of IgG complexes, to improve the specificity of a PF4–heparin IgG assay. The novel assay had superior specificity to the detection of PF4–heparin IgG by ELISA, and performed similarly to the HemosIL AcuStar HIT IgG-specific assay, a test in which high specificity has been reported previously [18]. The combined use of both the dimeric rsFc\(\gamma\)RIIa and HemosIL AcuStar HIT IgG-specific assays to assign HIT correctly identified some HIT samples that would have been false negative with the use of either assay alone. The detection of HIT antibodies with the dimeric rsFc\(\gamma\)RIIa assay adds a functional qualitative component to the assay that mechanistically underlies the possible improved specificity and utility.

Dimeric rsFc\(\gamma\)RIIa binding requires closely spaced pairs of antibodies, as occurs in ICs [14], and has been used to detect Fc\(\gamma\)R functional antibodies against influenza virus and HIV envelope proteins [14,15,23–28]. HIT pathogenesis requires Fc-mediated clustering of platelet Fc\(\gamma\)RIIa, but two SRA-positive samples were not detected with the dimeric rsFc\(\gamma\)RIIa-binding assay. Studies of HIT complexes have shown that the formation of the
PF4–heparin–IgG complex is dynamic and, importantly, the proximity of the IgG Fcs in the pathogenic complexes is influenced by the binding of heparin, and the epitope specificity and avidity of the bound IgG [11,12,29,30]. The failure of the dimeric rsFc\(\gamma RIIa\) assay to detect these two HIT samples suggests that the Fcs were not appropriately presented in the current ELISA format. Thus, the assay may need further optimization to a format that mimics the dynamic behavior of the PF4 complexes that cluster platelet FcγRIIa in vivo. Nonetheless, our proof-of-concept study has shown that the dimeric rsFc\(\gamma RIIa\) assay has the potential to be predictive of HIT and warrants further investigation.

This study has established a novel FcγRIIa probe as a powerful surrogate for evaluating HIT antibody functionality. The use of dimeric rsFc\(\gamma RIIa\) also offers the advantages that it is simple, scalable, and adaptable to any format, such as multiplexing [25] and flow cytometry.

Addendum

B. D. Wines, E. E. Gardiner, and P. M. Hogarth designed the study, reviewed data, and drafted the manuscript. B. D. Wines, S. Esparon, E. E. Gardiner, and C. W. Tan performed experiments. All authors analyzed and interpreted the data, and critically reviewed the manuscript.

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Disclosure of Conflict of Interests

P. M. Hogarth and B. D. Wines report receiving grants from the Australian Centre for HIV and Hepatitis Virology Research during the conduct of the study, and have a patent issued (to the Burnet Institute): ‘Binding assays and method for probing antibody function with fc binding multimers WO 2017/054033 A1’. S. Esparon reports receiving grants from the Australian Centre for HIV and Hepatitis Virology Research during the conduct of the study. R. Baker reports providing clinical trial support for Biogen Idec, Boehringer Ingelheim, Bayer, Shire, Pfizer, Daiichi Sankyo, Portola, Alexion Pharmaceuticals, Astellas, and CSL Behring, serving on clinical advisory boards of Bayer, Shire, Pfizer, and Amgen, and receiving research support from Bristol-Meyers Squibb, Shire, and Bayer. E. Duncan reports receiving personal fees from Novo Nordisk. The other authors state that they have no conflict of interest.

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