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BRIEF REPORT

Dimeric Fc γ R ectodomains detect pathogenic anti-platelet factor 4–heparin antibodies in heparin-induced thrombocytopenia

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Essentials

- Fc γ RIIa mediates life-threatening heparin-induced thrombocytopenia (HIT).
- Most anti-platelet factor (PF)4–heparin IgGs are not pathogenic so diagnosis of HIT is challenging.
- Dimeric rsFc γ RIIa was used to quantify receptor-binding activity of anti-PF4–heparin antibodies.
- Dimeric rsFc γ RIIa binding specifically correlated with occurrence of HIT.

Summary. *Background:* Heparin-induced thrombocytopenia (HIT) is a major and potentially fatal consequence of antibodies produced against platelet factor 4 (PF4)–heparin complexes following heparin exposure. Not all anti-PF4–heparin antibodies are pathogenic, so overdiagnosis can occur, with resulting inappropriate use of alternative anticoagulation therapies that have associated risks of bleeding. However, definitive platelet functional assays are not widely available for routine analysis. *Objectives:* To assess the utility of dimeric recombinant soluble Fc γ RIIa (rsFc γ RIIa) ectodomains for detecting HIT antibodies. *Patients/Methods:* Plasma from 27 suspected HIT patients were tested for pathogenic anti-PF4–heparin

antibodies by binding of a novel dimeric Fc γ RIIa ectodomain probe. Plasmas were also tested by the use of PF4–heparin IgG ELISA, the HemosIL AcuStar HIT IgG-specific assay, and a serotonin release assay (SRA). *Results:* The dimeric rsFc γ RIIa test produced no false positives and excluded four samples that were positive by IgG ELISA. In this small patient cohort, the novel assay correctly assigned 93% of the suspected HIT patients, with two of the HIT patients being scored as false negatives. The improved discrimination of the novel assay over the IgG ELISA, which scored four false positives, supports the mechanistic interpretation that binding of dimeric rsFc γ RIIa detects pairs of closely spaced IgG antibodies in PF4–heparin immune complexes. *Conclusions:* This study found the cell-free, function-based dimeric rsFc γ RIIa assay to be convenient, simple, and potentially predictive of HIT. The assay had improved specificity over the IgG ELISA, and correlated strongly with the AcuStar HIT IgG-specific assay, warranting further evaluation of its potential to identify HIT in larger patient cohorts.

Keywords: enzyme immunoassay; heparin; platelet factor 4; thrombocytopenia; thrombosis.

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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 Fc γ RIIa, which is the only Fc γ R on platelets, triggering their activation and aggregation, leading to thrombosis. Binding to Fc γ RIIa 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 Fc γ RIIa-dependent manner [8]; it causes HIT in a human Fc γ RIIa/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 Fc γ Rs.

Pathological HIT antibodies engage Fc γ RIIa, and trigger platelet activation and clearance [3] and tissue factor production [4]. The pathology depends, in part, on an R131H polymorphism within Fc γ RIIa, which does not alter the expression levels of the receptor but does significantly alter the affinity of Fc γ RIIa for its ligand [13]. We recently described the use of dimeric recombinant soluble Fc γ RIIa (rsFc γ RIIa) to determine the proximity of pairs of IgG antibodies in immune cell-activating ICs [14]. The binding of dimeric rsFc γ RIIa in this assay is correlated with the capacity of IgG ICs to activate Fc γ R-dependent cellular responses [14,15]. In this study, we tested the capacity of this unique dimeric rsFc γ RIIa 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 rsFc γ RIIa (His131 allelic form) has been described previously [14]. To assess the ability of dimeric rsFc γ RIIa 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 rsFc γ RIIa–biotin ($0.2 \mu\text{g mL}^{-1}$) 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 rsFc γ RIIa 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 rsFcγRIIIa (His131) to detect pathogenic anti-PF4–heparin antibodies was evaluated by comparison with these established assays.

The novel dimeric rsFcγRIIIa 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

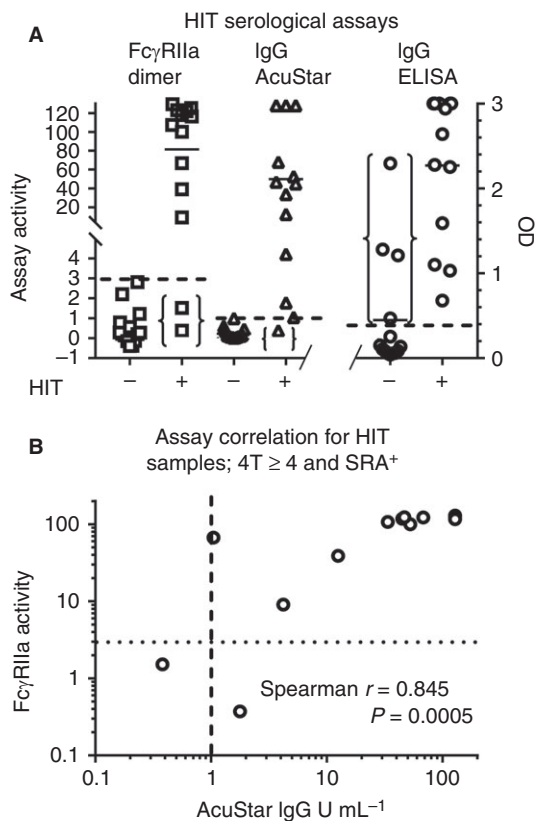


Fig. 1. Comparison of the novel dimeric recombinant soluble FcγRIIIa (rsFcγRIIIa) assay with existing serological assays for the detection of heparin-induced thrombocytopenia (HIT) antibodies. (A) Suspected HIT patients were segregated on the basis of a positive serotonin release assay (SRA) result and 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 rsFcγRIIIa 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 rsFcγRIIIa 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 rsFcγRIIIa 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 rsFcγRIIIa and AcuStar HIT IgG-specific assays. When HIT positivity is defined as rsFcγRIIIa positivity and/or AcuStar HIT IgG-specific assay positivity, sensitivity is improved over that with either assay used alone.

summarized in Table 1. The dimeric rsFcγRIIIa 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 rsFcγRIIIa 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 rsFcγRIIIa 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 rsFcγRIIIa assays appropriately assigned low, below cut-off, values to the SRA-negative samples, although some were very near the cut-off values of both assays. Furthermore, the AcuStar HIT IgG-specific and dimeric rsFcγRIIIa assays also correctly assigned as negative a sample (see filled blue star; Figure 2) that had $\sim 50\%$ 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 rsFcγRIIIa 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 rsFcγRIIIa assay-negative (0.4% response; cut-off

Table 1 Immunoassays of patient plasma ($n = 27$) with or without heparin-induced thrombocytopenia (HIT) defined by a positive serotonin release assay result and a 4Ts score of ≥ 4

Assay	Positive test		Negative test		Specificity	Sensitivity
	True	False	True	False		
Dimeric rsFcγRIIIa	11	0	14	2	1.00	0.85
PF4–heparin IgG ELISA	13	4	10	0	0.71	1.00
AcuStar HIT IgG-specific	12	0	14	1	1.00	0.92

PF4, platelet factor 4; rsFcγRIIIa, recombinant soluble FcγRIIIa.

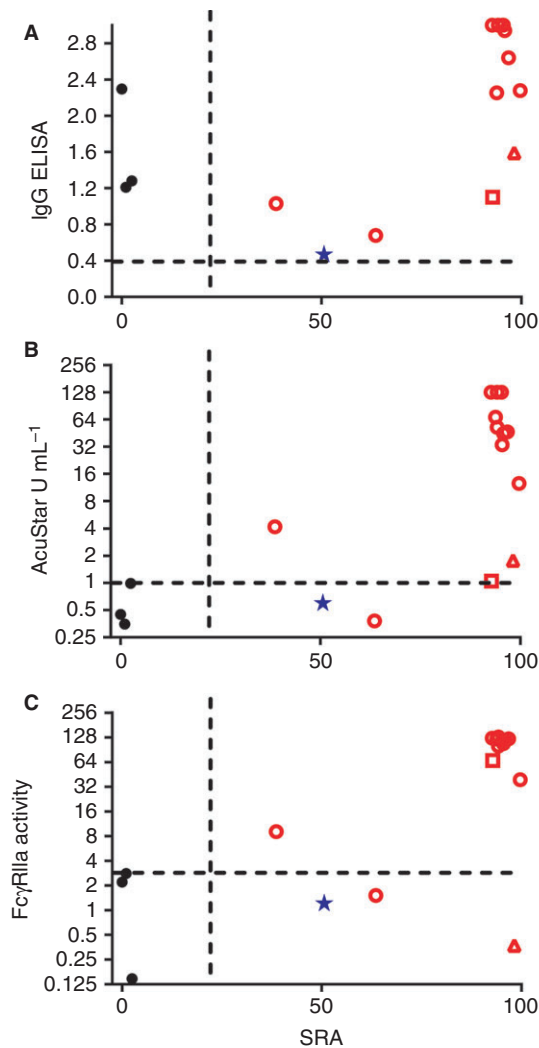


Fig. 2. Correlation of the novel dimeric recombinant soluble FcγRIIa (rsFcγRIIa) assay and the existing serological assays with serotonin release. Results from all the serological assays of samples positive in the IgG ELISA, including four HIT false positives (filled blue star and black circles), were compared against the SRA results. The SRA (release at 0.1 U mL^{-1} heparin) was compared with (A) the PF4-heparin IgG ELISA, (B) the AcuStar HIT IgG-specific assay, and (C) the novel dimeric rsFcγRIIa assay. The dashed lines indicate the cut-off values for a positive result with each assay. Open symbols represent HIT-diagnosed patients (positive SRA result and a 4Ts score of ≥ 4). Filled symbols represent PF4-heparin IgG ELISA-positive HIT-negative patients, with the blue star symbol indicating one SRA-negative patient with equivalent levels of serotonin release with both high-dose and low-dose heparin stimulation. The open triangle indicates an HIT patient plasma sample that was weakly AcuStar HIT IgG-specific assay-positive and dimeric rsFcγRIIa assay-negative, and the open square indicates an HIT patient plasma sample that was weakly AcuStar HIT IgG-specific assay-positive and strongly dimeric rsFcγRIIa assay-positive. [Color figure can be viewed at wileyonlinelibrary.com]

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 rsFcγRIIa assay (67% response; open square, Figure 2).

Thus, defining samples as HIT-positive according to AcuStar HIT IgG-specific assay-positive and/or dimeric rsFcγRIIa 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 FcγRIIa on platelets [1,3,5], and possibly other FcγRs on monocytes [4,19]. The size and stoichiometry of IgG ICs are major determinants of both the avidity of interaction with the low-affinity FcγRs [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 FcγRs. 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 rsFcγRIIa, a probe of the FcγR-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 rsFcγRIIa 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 rsFcγRIIa assay adds a functional qualitative component to the assay that mechanistically underlies the possible improved specificity and utility.

Dimeric rsFcγRIIa binding requires closely spaced pairs of antibodies, as occurs in ICs [14], and has been used to detect FcγR functional antibodies against influenza virus and HIV envelope proteins [14,15,23–28]. HIT pathogenesis requires Fc-mediated clustering of platelet FcγRIIa, but two SRA-positive samples were not detected with the dimeric rsFcγRIIa-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 rsFcγRIIa 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 rsFcγRIIa 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 rsFcγRIIa 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 2017054033 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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