A Novel PF4-Dependent Platelet Activation Assay Identifies Patients Likely to Have Heparin-Induced Thrombocytopenia/Thrombosis

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BACKGROUND: Almost without exception, patients with heparin-induced thrombocytopenia/thrombosis (HIT) have antibodies that recognize platelet factor 4 (PF4) in a complex with heparin; however, many heparin-treated patients without HIT are also antibody-positive. A platelet activation test, the serotonin release assay (SRA), is useful for identifying a subset of antibodies that are platelet-activating and most likely to cause HIT. However, this “gold standard” assay for HIT diagnosis is technically demanding and is routinely available only through referral laboratories, limiting its availability for timely diagnosis and management.

METHODS: We compared the diagnostic performance of the SRA with that of a technically simple platelet activation assay, the PF4-dependent P-selectin expression assay (PEA), which uses platelets pretreated with PF4 as targets for antibody detection. Archived serum samples from 91 patients for whom clinical information (HIT 4Ts [thrombocytopenia, timing of platelet count fall, thrombosis, and other causes of thrombocytopenia] score) was available were used. Patients with an intermediate 4Ts score and a PF4 ELISA (enzyme-linked immunosorbent assay) optical density ≥ 2.0 , or a high 4Ts score and a PF4 ELISA optical density ≥ 1.0 , were considered HIT positive; others were designated HIT negative.

RESULTS: The PEA had higher diagnostic accuracy (area under the curve, 0.92 vs 0.82; $P = .02$) than the SRA, using this definition of HIT. Eleven of 16 serum samples that were PEA positive and SRA negative were HIT positive. Studies done with identical target platelets and serially diluted samples from patients with HIT showed that the PEA is inherently more sensitive than the SRA for the detection of platelet-activating antibodies.

CONCLUSIONS: The PEA is technically less demanding than the SRA and may be more accurate for the diagnosis of HIT.

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ABBREVIATIONS: 4Ts = thrombocytopenia, timing of platelet count fall, thrombosis, and other causes of thrombocytopenia; AUC = area under the curve; GPIIb = glycoprotein IIb; HDH = high-dose heparin; HIT = heparin-induced thrombocytopenia/thrombosis; LDH = low-dose heparin; MFI = median fluorescence intensity; OD = optical density; PEA = PF4-dependent P-selectin expression assay; PF4 = platelet factor 4; PF4 ELISA = IgG-specific heparin (or polyvinyl sulfonate):PF4 enzyme-linked immunosorbent assay; ROC = receiver operating characteristic; SRA = serotonin release assay

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Despite improvements in diagnosis and treatment, heparin-induced thrombocytopenia/thrombosis (HIT) remains a major cause of morbidity and mortality in patients receiving heparin.¹⁻³ HIT is characterized by thrombocytopenia, usually beginning 5 to 10 days after starting heparin therapy. Although thrombocytopenia in patients receiving heparin is, in itself, generally mild, it can provide an important clue that a patient is developing HIT and is at risk for thromboembolic complications. Experts view HIT as a clinicopathological syndrome diagnosed most reliably on the basis of both clinical findings and antibody test results.⁴ Nearly all patients with HIT produce antibodies that recognize the platelet α granule chemokine platelet factor 4 (PF4) in a complex with heparin, or a similar negatively charged polyanion, and can be rapidly detected in immunoassays that use heparin (or polyvinyl sulfonate):PF4 complexes as targets (PF4 ELISA).⁵⁻⁸ The PF4 ELISA is highly sensitive for antibody detection and is useful because a negative test result argues strongly against the diagnosis of HIT. However, many

patients who produce positive test results in this assay never develop HIT.⁹

Pathogenic HIT antibodies usually produce positive results in an alternative “gold standard” diagnostic test, the serotonin release assay (SRA).^{9,10} However, the SRA is technically demanding and is available routinely only through a few reference laboratories, making it difficult to use test results for patient treatment decisions, which should be made as soon as possible after HIT is suspected. We have described a PF4-dependent P-selectin (CD62p) expression assay (PEA) that is simple to perform and can be completed within a few hours.¹¹ In this article, we describe studies done to evaluate the diagnostic utility of the PEA, using blood samples from a cohort of patients suspected of having HIT and whose clinical course was defined by communication with their physicians. Findings made indicate that the PEA is at least as accurate as the SRA for characterization of pathogenic heparin-induced antibodies and suggest that this relatively simple assay can facilitate early diagnosis and treatment of HIT.

Materials and Methods

Reagents

Unfractionated porcine heparin (Sagent Pharmaceuticals), bovine serum albumin (Sigma-Aldrich), phycoerythrin-labeled mouse anti-human P-selectin (BD Biosciences), and Alexa Fluor 647-labeled anti-glycoprotein IIb (GPIIb) antibodies (290.5; BloodCenter of Wisconsin) were used. Human PF4 was purified from normal donor platelets.¹²

HIT Samples and 4Ts Scores

Ninety-one patient serum samples submitted for testing because of a suspicion of HIT were from the HIT sample archive at the BloodCenter of Wisconsin. Seventy-four of these samples had been collected as part of a previous study¹³ in which results obtained in the IgG-specific polyvinyl sulfonate-PF4 ELISA (PF4 ELISA) test for HIT antibodies were compared with patients' clinical status as defined by the 4Ts (thrombocytopenia, timing of platelet count fall, thrombosis, and other causes of thrombocytopenia) score.¹⁴ Seventeen additional serum samples were from patients highly likely to have HIT, who were referred more recently for diagnostic testing, and for whom detailed clinical histories had been obtained. e-Table 1 summarizes 4Ts scores and PF4 ELISA results for both sets of samples. Clinical information used to define 4Ts

status was obtained by a physician-investigator experienced in HIT diagnosis in discussion with the referring physician, using a standardized script.¹³ A 4Ts score (ranging from 0 to 8)¹⁴ was assigned independently by each of two physicians after a review of clinical information, and a mean score was calculated. When physician scores differed by two points or more, consensus was reached in discussion between the scorers. Since clinical information was collected retrospectively, the contacted physicians were aware of prior HIT test results and the same was true of investigators collecting the information; however, every attempt was made to administer the survey uniformly without reference to prior serologic testing. About half of the patients had a low 4Ts score (mean score, < 4) and the remainder had either intermediate (mean score, 4 to < 6) or high (mean score, 6-8) 4Ts scores (e-Table 1).

PEA and SRA

Individuals performing SRA and PEA testing were blinded to the 4Ts scores and prior HIT test results. The PEA was performed as previously described.¹¹ Normal platelets were isolated from pooled, citrated platelet-rich plasma of two or three group O blood donors. Prostaglandin E₁ (50 mg/mL) was added and platelet-rich plasma was centrifuged at $150 \times g$ for 15 minutes. The supernatant was then centrifuged at $1,000 \times g$ for 15 minutes to pellet platelets. The platelet button was resuspended in phosphate-buffered isotonic saline (pH 7.2)-1% bovine serum albumin. These washed normal donor platelets (1×10^6) were first treated for 20 minutes at room temperature with (1) PF4 (37.5 μ g/mL), (2) PF4 (37.5 μ g/mL) plus unfractionated high dose heparin (HDH, 125 units/mL), or (3) phosphate-buffered isotonic saline (pH 7.2)-1% bovine serum albumin alone in a total volume of 40 μ L. Ten microliters of patient serum was then added and the mixture was incubated for 1 hour at room temperature without agitation. After addition of labeled anti-P-selectin and anti-GPIIb antibodies, platelet events were gated by GPIIb positivity, and P-selectin expression

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(median fluorescence intensity, MFI) was recorded. In addition to a normal serum “calibrator,” known positive and negative patient samples were included in each run. Maximum P-selectin expression (100%) was measured by treating platelets with thrombin receptor-activating peptide (TRAP, 25 µg/mL). Results were expressed as the percentage of maximum P-selectin expression corrected for background signal obtained with normal serum as follows:

$$\text{Percent activation} = \frac{\text{Sample MFI} - \text{Normal serum MFI}}{\text{TRAP MFI} - \text{Normal serum MFI}} \times 100$$

The SRA was performed as described by Sheridan et al¹⁵ with slight modifications. Sera were considered positive in the SRA if serotonin release was $\geq 20\%$ with low-dose heparin (LDH) and $< 20\%$ with HDH, and there was $\geq 8\%$ difference between LDH and HDH samples.

Results

PEA Has High Diagnostic Accuracy

The positive predictive value of an intermediate 4Ts score, a high 4Ts score, or a PF4 ELISA optical density (OD) ≥ 1.0 when used alone for HIT diagnosis is modest at 14%, 64%, and 42%, respectively.^{17,18}

Accordingly, experts in the field recommend use of PF4 ELISA OD ≥ 2.0 or ≥ 1.0 together with an intermediate or high 4Ts score (4-5 or 6-8, respectively) to optimize diagnosis.^{19,20} However, a low 4Ts score, in itself, has a high negative predictive value for HIT.^{17,21}

To increase diagnostic stringency for the purpose of this study, we considered a patient to have clinical HIT (ie, to be “HIT positive”) if they had an intermediate 4Ts score and a PF4 ELISA OD ≥ 2.0 , or a high 4Ts score and a PF4 ELISA OD ≥ 1.0 . All other patients were designated “HIT negative.” Table 1 summarizes the demographics of patient groups classified in this way. By design, ELISA OD and 4Ts scores were higher in the HIT-positive group (Table 1), but the two groups were otherwise comparable. In line with expectations, only three of 43 patients with low 4Ts scores had a PF4 ELISA OD ≥ 1.0 while nine of 23 patients with intermediate 4Ts scores and 21 of 25 patients with high 4Ts scores had PF4 ELISA OD ≥ 1.0 (e-Fig 1).

The PEA and the SRA were performed on each of the 91 serum samples. ROC analysis provides a way to assess the relationship between cost (false positives and negatives) and benefit (true positives and negatives) in diagnostic decision-making²² and was performed on the dataset (Fig 1). The AUC, a measure of “accuracy” reflecting the likelihood that a test will correctly discriminate between patients with and without disease, was significantly greater for the PEA than for the SRA

Statistical Analysis and Ethics Approval

Mann-Whitney and Fisher exact tests were used to compare continuous and categorical variables, respectively. Receiver operating characteristic (ROC) curves (Analyse-it Software, Ltd) were constructed for accuracy analysis. Areas under the curve (AUCs) were compared by the method of DeLong et al.¹⁶ Diagnostic “accuracy” is a measure of the ability of a test to discriminate between diseased and unaffected individuals. Diagnostic “sensitivity” refers to the ability of an assay to correctly identify patients with disease and was calculated by dividing the number of assay-determined true positives by the total number of disease positives. Diagnostic “specificity” measures the ability of an assay to correctly identify patients without disease and was calculated by dividing the number of assay-determined true negatives by the total number of disease negatives. A *P* value less than .05 was considered significant. Studies were approved by the Institutional Review Board of the Medical College of Wisconsin (Protocol PRO00023318).

(0.92 vs 0.82; *P* = .02), using our definition of HIT. In a setting such as HIT, where both the sensitivity and specificity of a diagnostic assay are important, one way to define a threshold for positivity is to determine the point on the ROC curve where the sum of sensitivity and specificity is maximized.²² This point in the PEA ROC curve corresponds to $\geq 24\%$ P-selectin expression (Fig 1, arrow). Therefore, samples meeting this criterion were considered to be positive in the PEA, provided

TABLE 1] Patient Characteristics

	HIT Positive ^a (n = 26)	HIT Negative ^b (n = 65)	<i>P</i> Value
Age, y			
Range	41-86	12-94	NS
Mean (SEM)	63 (2)	62 (2)	
Sex			
Male, No. (%)	12 (46)	39 (60)	NS
Female, No. (%)	14 (54)	26 (40)	
Medical vs surgical			
Medical, No. (%)	19 (73)	41 (63)	NS
Surgical, No. (%)	7 (27)	24 (37)	
PF4 ELISA OD			
Range	1.0-4.7	0.1-2.3	< .0001
Mean (SEM)	2.3 (0.2)	0.4 (0.1)	
4Ts score			
Range	4.5-8	0-8	< .0001
Mean (SEM)	6.4 (0.2)	3 (0.2)	

4Ts = thrombocytopenia, timing of platelet count fall, thrombosis, and other causes of thrombocytopenia; ELISA = enzyme-linked immunosorbent assay; HIT = heparin-induced thrombocytopenia/thrombosis; NS = not significant; OD = optical density; PF4 = platelet factor 4.

^aHIT positive: Intermediate 4Ts score and a PF4 ELISA OD ≥ 2.0 , or a high 4Ts score and a PF4 ELISA OD ≥ 1.0 .

^bHIT negative: All other patients.

Figure 1 – The PEA demonstrates higher diagnostic accuracy for heparin-induced thrombocytopenia/thrombosis than the SRA. Receiver operating characteristic curve analysis was performed, designating patients with intermediate 4Ts (thrombocytopenia, timing of platelet count fall, thrombosis, and other causes of thrombocytopenia) scores and PF4 ELISA ≥ 2.0 or high 4Ts scores and PF4 ELISA ≥ 1.0 as disease positive. The PEA and SRA receiver operating characteristic curves are depicted by red and blue lines, respectively. The arrow denotes the point on the PEA curve that maximizes the sum of diagnostic sensitivity and specificity. The area under the curve was 0.92 for the PEA and 0.82 for the SRA ($P = .02$). PEA = PF4-dependent P-selectin expression assay; PF4 ELISA = IgG-specific heparin (or polyvinyl sulfonate):PF4 enzyme-linked immunosorbent assay; SRA = serotonin release assay.

P-selectin expression was inhibited at least 50% by HDH. Accuracy of the PEA defined by ROC analysis was superior to that of the SRA even when the criterion for a positive PF4 ELISA result was lowered to an OD

of 1.0 or 0.4 for patients with intermediate-to-high 4Ts scores (AUC: 0.93 [PEA] vs 0.82 [SRA], $P < .05$; and 0.86 [PEA] vs 0.75 [SRA], $P < .05$ for PF4 ELISA OD cutoffs of ≥ 1.0 and ≥ 0.4 , respectively; data not shown). The same was true if patients with HIT-positive results were defined as having a high 4Ts score (6-8) without consideration of PF4 ELISA results (0.87 [PEA] vs 0.72 [SRA]; $P < .01$; data not shown). In the SRA¹⁵ and in HIT assays based on annexin V binding²³ and platelet activation/aggregation,²⁴ platelet-activating antibodies are detected in a reaction mixture that includes LDH. To evaluate whether accuracy of the PEA is enhanced by LDH, the 91 patient samples were tested in the PEA with added LDH (0.125 units/mL). Results showed that LDH does not influence test accuracy (AUC, 0.92 [PEA] vs 0.93 [PEA-LDH]; $P =$ not significant; data not shown).

PEA Detects Nearly All HIT Positives

Figure 2A shows that 25 of 26 HIT-positive patient serum samples induced P-selectin expression to at least 24%, confirmed by inhibition with HDH. One HIT-positive sample negative in the PEA had a P-selectin expression level of 17%. The SRA was positive with only 14 of the 26 HIT-positive samples, all of which were confirmed with HDH. Among 65 HIT-negative patient serum samples, the SRA and PEA were confirmed positive in five and 10 cases, respectively (Fig 2B). Three HIT-negative sera that induced P-selectin expression $\geq 24\%$ did not inhibit with HDH (asterisks, Fig 2B). e-Figure 2 illustrates the importance of preincubating

Figure 2 – The PEA detected 25 of 26 heparin-induced thrombocytopenia/thrombosis (HIT)-positive patient samples. A and B, The PEA and SRA were performed on HIT-positive (A) and HIT-negative (B) samples. Solid circles (P-selectin expression) depict the average of duplicate measurements and open circles depict single SRA test results. Values on the ordinate indicate platelet activation (percentage of maximum signal as described in Materials and Methods) obtained with the SRA and PEA. Solid lines connect the SRA and PEA results of each sample. Samples that were not inhibited $\geq 50\%$ in the presence of high-dose heparin are indicated by an asterisk. Horizontal arrows at the right and left of each panel indicate median results obtained with the PEA and SRA, respectively. The solid and dotted horizontal lines depict positive cutoffs of the SRA and PEA, respectively. See Figure 1 legend for expansion of abbreviations.

TABLE 2] Clinical and Serological Characteristics of PEA⁺/SRA⁻ Patients: PEA⁺/SRA⁻ HIT-Negative Patients

Patient No.	Mean 4Ts Score (Physician Investigator)	Suspicion for HIT (Referring Physician)	Age, Sex, y	Surgical (S) or Medical (M) Setting (Surgical Detail, if Applicable)/ Type of Heparin	TP (% of Baseline); Timing	Thrombosis (New, Worsening, or None)	Other Causes of TP	Alternative Anticoagulation/ Outcome and Comments	IgG PF4 ELISA (OD)/SRA (%) / PEA (%)
1	1.0	Medium	72, M	S (cardiac pacemaker placement)/UFH	< 30; unclear timing	None	DIC (unclear cause)	None/Death due to underlying cardiac cause	0.7/0/53
2	1.5	Low	61, M	S (CABG)/UFH	30-50; 3 d	None	Infection; cardiopulmonary bypass	None/death due to CHF	0.4/1/74
3	2.5	Low	72, F	M/LMWH	> 50; 3 d	None	s/p BMT	None/Hospitalized at last follow-up	0.3/1/85
4	2.5	Low	61, M	S (AAA repair)/ UFH	> 50; 3 d	None	Possibly related to procedure	None/Discharged home	1.7/14/99
5	4.5	Medium	46, M	S (liver surgery)/ UFH	30-50; ≤ 16 d (was outpatient, hence CBC was not monitored daily)	New (hepatic artery thrombosis)	Hepatic surgery/ malignancy	None/Discharged; being treated for underlying malignancy	0.9/0/38

AAA = abdominal aortic aneurysm; CABG = coronary artery bypass grafting; CHF = congestive heart failure; DIC = disseminated intravascular coagulation; F = female; LMWH = low-molecular-weight heparin; M = male; PEA = PF4-dependent P-selectin expression assay; s/p BMT = status post-bone marrow transplantation; SRA = serotonin release assay; TP = thrombocytopenia; UFH = unfractionated heparin. See [Table 1](#) legend for expansion of other abbreviations.

TABLE 3] Clinical and Serological Characteristics of PEA⁺/SRA⁻ Patients: PEA⁺/SRA⁻ HIT-Positive Patients

Patient No.	Mean 4Ts Score (Physician Investigator)	Suspicion for HIT (Referring Physician)	Age, Sex, y	Surgical (S) or Medical (M) Setting (Surgical Detail, if Applicable)/Type of Heparin	TP (% of Baseline); Timing	Thrombosis (New, Worsening, or None)	Other Causes of TP	Alternative Anticoagulation/ Outcome and Comments	IgG PF4 ELISA (OD)/SRA (%) / PEA (%)
1	4.5	High	86, F	M/UFH	> 50; 4 d	None	None	Lepirudin/ Discharged home	2.8/2/46
2	6.0	High	60, M	M/UFH	> 50; 1 d with recent heparin exposure (< 30 d)	None	None	Argatroban/ Discharged home	1.4/0/24
3	6.0	High	63, F	M/UFH	> 50; 5 d	None	None	Aspirin/Discharged home. Heparin-free dialysis for underlying ESRD	1.5/2/53
4	6.0	High	65, F	M/UFH	> 50; 7 d	None	None	Argatroban/ Discharged home. Heparin-free dialysis for underlying ESRD	1.5/16/98
5	6.0	Medium	74, M	M/UFH and LMWH	> 50; 2 d with recent heparin exposure (< 30 d)	None	None	Fondaparinux/ Death due to advanced malignancy	1.6/4/99
6	6.0	High	61, F	M/UFH	> 50; 10 d	New (multiple extensive thrombosis sites not documented)	None	Argatroban/ Developed ICH while taking argatroban. Final disposition unknown	2.1/4/102
7	6.0	High	73, M	S (nephrectomy)/ UFH	> 50; 1 d with recent heparin exposure (< 30 d)	None	None	Inadequate information/ Recovered. Patient taking warfarin at time of last follow-up	2.2/5/105
8	6.5	High	56, F	S (bilateral sphenoidotomies)/ LMWH	> 50; 8 d	New (PE)	Possible infection/ antibiotic use	Argatroban/ Transferred to rehabilitation facility	1.1/1/33

(Continued)

TABLE 3] (Continued)

Patient No.	Mean 4Ts Score (Physician Investigator)	Suspicion for HIT (Referring Physician)	Age, Sex, y	Surgical (S) or Medical (M) Setting (Surgical Detail, if Applicable)/Type of Heparin	TP (% of Baseline); Timing	Thrombosis (New, Worsening, or None)	Other Causes of TP	Alternative Anticoagulation/ Outcome and Comments	IgG PF4 ELISA (OD)/SRA (%)/PEA (%)
9	6.5	High	67, M	M/UHF	> 50; 2 d with recent heparin exposure (< 30 d)	None	None	Argatroban, fondaparinux/ Discharged home	1.7/2/31
10	7.5	Medium	56, F	M/UHF	> 50; 4 d	New (LE superficial venous thrombosis)	None	Fondaparinux/ Recovered. Transferred to rehabilitation facility	2.1/1/78
11	8.0	High	64, F	M/UHF	> 50; ≤ 14 d (was outpatient, hence CBC was not monitored daily)	New (multiple PEs, lower extremity DVT)	None	Argatroban/Still in hospital at last follow-up	2.2/7/101

ESRD = end-stage renal disease; LE = lower extremity; PE = pulmonary embolism. See Table 1 and 2 legends for expansion of other abbreviations.

platelets with PF4 for use in the PEA by showing that P-selectin expression is minimal in the absence of PF4, with median P-selectin expression of 6% and 3% in HIT-positive and HIT-negative samples, respectively.

PEA Has High Diagnostic Sensitivity/Specificity

Among the 91 samples tested, the PEA was positive in 16 samples that were SRA negative (Figs 2A, 2B). However, none of the PEA-negative samples were SRA positive (Figs 2A, 2B). Of the 16 patients providing samples that were PEA positive and SRA negative (PEA⁺/SRA⁻), two-thirds (11) had clinical disease (HIT positive) based on the definition of HIT used and 5 were HIT negative. Tables 2 and 3 summarize clinical and laboratory characteristics of the five PEA⁺/SRA⁻ HIT-negative patients and the 11 PEA⁺/SRA⁻ HIT-positive patients, respectively. Among those who were HIT negative, referring physicians had a low suspicion of HIT in three of five patients and a medium level of suspicion in the remaining two (Table 2). Notably, one PEA⁺/SRA⁻ HIT-negative patient (patient 4; Table 2) had a PF4 ELISA OD of 1.7, and a borderline-positive SRA of 14% (inhibited to 0% with HDH). Referring physicians had a high level of clinical suspicion for HIT in nine of 11 HIT-positive patients (Table 3). One HIT-positive patient (patient 4; Table 3) gave a borderline positive result in the SRA (16%; inhibited to 1% with HDH). By definition, all 16 PEA⁺ samples were inhibited at least 50% with HDH in the PEA. The diagnostic sensitivity (fraction of HIT-positive patient samples detected) of the PEA in this cohort was thus 96% compared with 54% for the SRA (Fig 3). The specificities (fraction of HIT negatives correctly identified as negative) of the PEA and SRA tests were comparable at 85% (PEA) and 92% (SRA), respectively (Fig 3).

PEA Detects Low-Titer Platelet-Activating Antibodies

All SRA-positive sera (14 HIT positive, five HIT negative) and 16 of 72 SRA-negative sera (11 HIT positive, five HIT negative) were positive in the PEA, suggesting that the PEA has higher analytical sensitivity than the SRA for detecting platelet-activating HIT antibodies. To investigate this further, three serum samples, from patients with high 4Ts scores and positive SRA results (two with thrombosis and one with isolated thrombocytopenia), were tested simultaneously in both assays at various dilutions, using the same target platelets. As shown in Figures 4A-4C, the PEA produced positive results at serum dilutions four to more than 16 times greater than those required to

Figure 3 – The PEA has high diagnostic sensitivity and specificity. Paired columns denote diagnostic sensitivity (left) and specificity (right) of the SRA and PEA in the 91 patients studied. The sensitivity and specificity of the PEA were 96% and 85%, respectively, compared with a sensitivity and specificity of 54% and 92%, respectively, for the SRA. Error bars represent 95% CIs. See Figure 1 legend for expansion of abbreviations.

produce a positive test result in the SRA. The specificity of each reaction was confirmed with HDH. Together, these observations suggest that the greater analytical sensitivity of the PEA for detecting platelet-activating HIT antibodies accounts for its greater diagnostic accuracy and its ability to detect a subset of patients who are SRA negative and HIT positive.

Discussion

A commonly used, technically simple assay, the PF4 ELISA, has low diagnostic specificity for HIT,^{9,25} particularly at an OD cutoff of 0.4. A functional assay, the SRA, is much more specific but, owing to its complexity, is not routinely available on a timely basis so that results can impact initial diagnosis and treatment. Overdiagnosis of HIT has important medical and economic consequences and underdiagnosis places patients at risk for thrombosis.²⁶⁻³⁰

Antibody detection by the PF4-augmented PEA involves preferential recognition of platelet-bound PF4 by platelet-activating pathogenic HIT antibodies, leading to platelet activation through CD32, the platelet receptor for the IgG Fc domain.^{11,31} The present study was designed to assess the ability of the PEA to selectively identify patients considered to be HIT positive on the basis of both clinical presentation (intermediate to high 4Ts score of 4-8) and PF4 ELISA results ($OD \geq 2.0$ or ≥ 1.0 , depending on the 4Ts score) as recommended.^{19,20,29} Using this classification, the diagnostic accuracy of the PEA was found to be significantly better than that of the SRA (AUC, 0.92 vs 0.82; $P < .05$) and the P-selectin expression level

of 24% was found to be optimal as a threshold for defining a positive test result. A significant difference in accuracy between the PEA and SRA was maintained

Figure 4 – The PEA has higher analytical sensitivity for detection of platelet-activating HIT antibodies. A-C, Three high 4Ts-scoring platelet-activating HIT sera were adjusted to the fold dilutions shown on the abscissa. The SRA and PEA were performed on these samples, using the same target platelets. PEA results (solid triangles) and SRA results (solid inverted triangles) are expressed on the ordinate. Individual points represent the average of duplicate determinations. Open triangles (PEA) and open inverted triangles (SRA) depict high-dose heparin control samples. The solid and dotted lines represent positive cutoffs of the SRA and PEA, respectively. See Figure 1 and 2 legends for expansion of abbreviations.

even if the PF4 ELISA OD threshold for positivity was lowered to the conventional value of 0.4, or was eliminated altogether by considering only patients with high 4Ts scores (6-8) to be HIT positive. Among 65 HIT-negative samples tested, five and 10 were positive in the PEA and SRA, respectively. The ten false-positive reactions obtained with the PEA are likely a consequence of its greater sensitivity for detection of platelet-activating antibodies. Use of dual criteria for defining HIT positivity (PF4 ELISA OD ≥ 1.0 or ≥ 2.0 and a 4Ts score of 4-8, as described) prevented any of the 10 patients from being so classified.

The greater sensitivity of the PEA for detection of platelet-activating antibodies in patients receiving heparin treatment appears to be the result of PF4-dependent priming of test platelets by a mechanism not yet fully defined, leading to enhanced antibody binding and activation via the platelet Fc receptor CD32.^{11,31} Interestingly, a recent report indicates that use of PF4-treated platelets also increases the sensitivity of the SRA for antibody detection.³² On the basis of three low 4T-scored patients with positive PF4-enhanced SRA results but negative SRA results, the report suggested that PF4-augmented SRA testing may not increase the rate at which clinically relevant antibodies are detected in that assay.³² Our findings indicate that the PEA does increase the rate at which pathogenic HIT antibodies are detected, since the majority (11 of 16) of the patients who tested SRA negative/PEA positive had HIT, using a stringent definition of disease positivity (Tables 2 and 3). A possible explanation for this finding—that the SRA as practiced by us is relatively insensitive—is unlikely since the SRA positivity rate in our high 4Ts-scored samples was 44%, similar to that reported recently by another large SRA referral laboratory (37%).³³ An interlaboratory study could help to resolve this issue.

Important limitations to this study include limited sample size, and notwithstanding the fact that every attempt was made to obtain clinical history without reference to prior serologic testing, treating physicians and investigators were aware of HIT serologic results at the time of 4Ts scoring. An additional limitation is that testing was not performed in real time as new samples were received for HIT diagnostic testing. As a referral laboratory not privy to clinical information on referred samples, this would have been difficult to do. Also, despite the fact that the archived samples studied had been assigned 4Ts scores for purposes completely unrelated to the PEA¹³ before this study was conceived, unintended selection biases for or against the PEA in sample selection cannot be ruled out. It should be noted, however, that the PF4 ELISA positivity rate (OD ≥ 0.4) among the 91 samples studied (51%; e-Table 1) closely approximates the rate obtained with patient samples submitted to our laboratory for PF4 ELISA testing over a consecutive 4-month period (44%), indicating a similar HIT serologic profile (B. R. Curtis, PhD, written communication, July 12, 2013).

Using a stringent definition of HIT positivity, our findings indicate that the PEA is at least comparable and may be superior to the gold standard SRA for detection of platelet-activating, presumptively pathogenic heparin-induced antibodies. Technical advantages of the PEA over the SRA are its relative simplicity, enabling it to be performed rapidly by any laboratory capable of doing flow cytometric assays; however, access to PF4 could be a challenge, especially for smaller laboratories. Thus, the PEA appears to have the potential to facilitate more timely and accurate identification of heparin-treated patients who develop HIT. Further laboratory and clinical studies to define more fully the extent to which its use can improve HIT treatment are indicated.

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