A Flow Cytometric Assay of Platelet Activation Marker P-Selectin (CD62P) Distinguishes Heparin-induced Thrombocytopenia (HIT) from HIT with Thrombosis (HITT)

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Key words
Heparin-induced thrombocytopenia, thrombosis, thrombocytopenia, drug-induced antibodies, heparin, platelet activation, P-selectin, CD62, CD36

Summary
Heparin induced thrombocytopenia (HIT) is a well-known complication of heparin administration but usually resolves upon discontinuation without sequelae. However, a small proportion of HIT patients develop thrombosis associated with HIT, designated as HITT, which is often life-threatening and may lead to gangrene and amputations. Existing laboratory methods of confirming HIT/HITT do not distinguish between HIT and HITT. We report a flow cytometric assay of platelet activation marker CD62P to distinguish the effects of addition of HIT vs. HITT plasma to normal blood. Briefly, normal whole blood was incubated with platelet-poor plasma from 12 patients with HIT, 30 with HIT, and 65 controls, in presence and absence of heparin, and expression of CD62P was assayed by flow cytometry. When the ratios of fluorescent intensity of CD62P with heparin divided by that without heparin were compared, HITT plasma induced significantly higher ratios than HIT plasma (HITT ratios ~2.5 vs. HIT ratios ~1.2; p < 0.001). Eleven of 12 HITT patients were positive by this test but only 5 of 30 HIT patients were positive (p < 0.0005). In a case of HIT with silent thrombosis, this assay gave a positive results prior to clinically evident thrombosis. In conclusion, this method distinguishes HITT from HIT and may be clinically useful in the detection of HITT, allowing early intervention for preventing catastrophic thrombosis.

Introduction
Heparin induced thrombocytopenia (HIT) is a relatively common drug-induced thrombocytopenia (1-3). Two types have been described (4-7): type I thrombocytopenia is mild and transient, developing within a few days of heparin administration and reversing while continuing heparin. It is not immunologic in origin and is not associated with thrombosis. Type II thrombocytopenia is immune-mediated, more severe and seldom reverses without discontinuation of heparin. It usually develops 3-15 days after initiating heparin therapy, or sooner if the patient was previously sensitized. Although most cases of type II are asymptomatic, a fraction of these patients develop thrombosis (1-3, 8), designated HIT-with-thrombosis (HITT), a much more serious complication leading to life-threatening venous and arterial thrombosis with high mortality, and often requiring amputation of extremities due to gangrene (1-3). The present study was limited to investigation of type II of heparin induced thrombocytopenia.

Early detection of HITT is critical in preventing catastrophic complications and preserving extremities from ischemic injury. Because of the wide use of heparin, HIT is frequently seen but there has been no laboratory assay for differentiating the dangerous HITT from the relatively innocuous HIT.

The target antigen for HIT antibodies was first identified by Amiral et al. (9). They found that these antibodies are directed against the complex of heparin with platelet factor 4 (PF4:heparin) (10, 11). Those observations have been confirmed by other investigators (12-14). It was shown that the formed immune complexes bind to the FcyRII receptor of platelets, leading to platelet destruction in HIT, and/or platelet activation resulting in thrombotic complications in HITT (15-17). Laboratory tests have been developed to detect heparin-dependent antibodies. Two types of tests have been devised, first, detection of PF4:heparin antibody, usually by binding of the antibody to PF4:heparin complexes fixed to the ELISA plate (10). The second type is based on functional activation of platelets by immune complexes of PF4:heparin, chiefly by assay of 14C serotonin release (18), by ATP release (19), PMP release assay (20), or platelet aggregation (21, 22). Although these tests have been used extensively they do not discriminate HITT from HIT.

We have applied a flow cytometric whole-blood assay of CD62P (P-selectin) to study activation of normal platelets induced by addition of platelet-poor plasma (PPP) from patients with HIT and HITT. CD62P is a constituent of alpha granules and is released to the platelet surface upon activation, a process parallel to secretion of serotonin (23). We examined CD62P expression following incubation of normal whole blood with plasma from HITT and HIT patients, and observed a significant difference in its induced expression between these two groups. Preliminary results were reported in (24, 25).

Patients, Materials and Methods
Materials. FITC-conjugated anti-CD41 was obtained from Coulter-Immuno-tech Inc., Hialeah, FL (Cat #PN IM0689). PE-conjugated anti-CD62P was purchased from Becton & Dickinson Inc., San Jose, CA (Cat #348107). Fc-specific goat anti-human IgG labeled with horseradish peroxidase was obtained from Sigma Immunochemicals, St. Louis, MO (Cat #AO170) and was used with TMB chromogenic substrate from the same company (Cat #T-8540). Lyophilized human platelet factor 4 was obtained from Sigma Chemicals Inc., St. Louis, MO (Cat #F-1385), as was heparin absorbent (Cat #H-9895). Bovine serum albumin, Fraction V, was obtained from ICN Immuno-Biologicals, Lisle, IL (Cat #B10033). The heparin used was usually a sample of that which was provided for the patient.
the patient actually received; if that was not available, porcine heparin was used, supplied as a 1 ml vial of 10,000 units, from Elkins-Sinn Co., Cherry Hill, N.J. (Cat #A-0410G). Fetal calf serum was obtained from GIBCO, Chagrin Falls, OH (Cat #2006140).

Patient population. We studied 42 patients with clinically diagnosed heparin induced thrombocytopenia at this university medical center, from 1995 to 1997. The following clinical criteria were used for diagnosis of HIT: (i) new onset of thrombocytopenia with reduction of platelet count > 50% and counts <120,000 in 4-22 days of initiating heparin therapy, (ii) there was no other known cause accounting for the new onset of thrombocytopenia (such as infection, new medications, etc.), (iii) thrombocytopenia was not transient, persisting through at least 2 separate examinations. Patients who developed mild transient thrombocytopenia at initiation of heparin therapy were defined as type I HIT and were excluded from the study. The 42 patients with HIT were studied along with 65 healthy controls. The HIT group consisted of 12 patients who developed HIT as described above but associated with new onset of thrombotic complications. Thrombosis was clinically evident in all patients with HIT except one, and was documented by radiologic or ultrasound studies. In the one exception, thrombosis was not detected clinically but doppler studies revealed new venous thrombosis. All HIT or HITT patients received unfractionated heparin, except one HIT patient who received low molecular weight heparin. Of the 12 HITT patients, 6 developed gangrene of affected extremities, 2 had arterial thrombosis (both CVA); 4 had venous thrombosis (3 with clinically evident venous thrombosis, one with silent small venous thrombosis detected by doppler study).

Sample collection. Blood was collected in blue-top B.D. Vacutainers™ (citrate anticoagulant) using a 21 gauge butterfly. The first tube collected was discarded or used for other purposes. Normal donors were always type O. Platelet-rich plasma (PRP) was prepared by centrifuging whole blood for 10 min at 160 g and platelet-poor plasma (PPP) by centrifuging PRP at 900 g for 6 min. If the patients on heparin within 48 h, the heparin was removed by "Heparinadsorbent" (Sigma, see Materials) according to manufacturer’s directions. This was required in 3 of 12 HITT and 5 of 30 HIT patients. We tested the effect of heparin adsorbent in this cases, and found that it consistently reduced CD62P expression in (-) heparin samples but had little effect on (+) heparin samples. We also tested about 4 heparin-free samples by deliberate addition of heparin, followed by heparin adsorbent and observed no change in positivity by our flow cytometric assay. Similar results were reported in (26): heparin adsorbent was used to eliminate spontaneous aggregation but did not interfere with response to heparin serum.

Flow cytometry. To 50 μl fresh whole blood from a normal O positive donor in 10 × 75 mm polypropylene tubes was added 100 μl of patient or control PPP, then 5 μl PF4 (20 μg/ml final) and 17 μl of heparin (diluted to yield 0.0, 0.1, and 10.0 U/ml final) and incubated for 10 min. Then 4 μl FITC-α-CD41 antibody and 4 μl phycoerythrin (PE) α-CD62P was added and incubated with an additional 10 min. Finally, an equal volume (180 μl) of 4% p-formaldehyde was added to each tube for fixation and to promote lysing of RBC. After exactly 10 min, additional, 1.0 ml phosphate buffered saline (PBS) pH 7.3 was added. After at least 45 min to allow lysis of RBC by p-formaldehyde, the samples were measured on a Coulter Profile II flow cytometer as described in (27, 28). All incubations were at room temperature on an orbital platform shaker at 120 rpm.

Calculation of results. Results are expressed as the ratio of fluorescent intensity of CD62P in the presence/absence of 0.1 U/ml heparin. Each of the three platelet populations (see Fig. 1) was analyzed for expression of activation marker CD62P; the number of CD62P(+) events, n1, n2, n3, in each histogram is multiplied by the mean fluorescent intensity of that population F1, F2, F3, giving the total mean fluorescence, Ffin:

\[ F_{\text{fin}} = n_1 F_{1} + n_2 F_{2} + n_3 F_{3}. \]

This number was divided by the total events, N (activated + non-activated from all 3 histograms) to give final mean fluorescence, Ffin:

\[ F_{\text{fin}} = F_{\text{fin}} / N. \]

The ratio R of this value in the presence/absence of heparin (0.1 U/ml) is the measure reported:

\[ R = \frac{F_{\text{fin}}}{F_{\text{fin}}}. \]

Positivity is defined as R > 1.5, as explained in the Results section.

ELISA method for assay of anti-heparin antibodies: The 96-well plates were coated with 50 μl of PF4/Heparin (0.6 μg/ml PF4 with 0.1 U/ml heparin) overnight at 4°C, according to the method of (29). The wells were then washed 3X with PBS (pH 7.2) containing 0.05% v/v Tween 20 (PBS-Tween). The plate was blocked with 250 μl of PBS-Tween/2% BSA at room temperature for 2 h, then washed 3X with PBS-Tween. The patient or normal control plasma was diluted 1:4 with PBS-Tween/2% BSA, then 100 μl were added to the wells and incubated 1 h at room temperature. After incubation the plate was washed 3X with PBS-Tween, then peroxidase-conjugated goat anti-human IgG (Fc specific; diluted 1:2000 in PBS/2%BSA) was added. After 1 h incubation at room temperature the plate was washed 3X with PBS-Tween, then 100 μl of TMB substrate buffer was added to each well. After 30 min the reaction was stopped by addition of 100 μl of 0.5 M H2SO4, then OD450nm were read on a plate reader (SLT LabInstruments Spectra). A result was considered positive for heparin:PF4 antibody if the ratio, (patient OD/pooled control OD) > 2.0. This cutoff was defined by a value > 2s above normal controls.

![Fig. 1](image_url) Representative example of flow cytometry results. The upper panels are plots of log forward scatter (LFS) vs. log side scatter (LSS). Three platelet populations were identified based upon these two parameters: free platelets (bitmap 3), small platelet aggregates (bitmap 2), and large platelet aggregates (bitmap 1). The lower panels show 2-color histograms of CD62P fluorescence (LF2) vs. CD41 fluorescence (LF1). The fluorescence data were derived from the free platelet population (bitmap 3) of respective upper panels. Events above the horizontal line in the lower panels (within region #2) are positive (+) for CD62P (p-selectin), i.e., show activation, as indicated. Additional details are provided in the Methods section and in Results.
Statistical analysis. To justify the use of ratios of CD62P expression in the presence vs. absence (±) of heparin, we confirmed that the relation between ± heparin results were both linear and through the origin in all three groups. When comparing differences between mean ratios, the level of significance was determined by Student’s t-test for unpaired variables. The chi-square test was used to determine statistical significance of the difference in frequency of positive outcomes between HITT and HIT populations.

Results

An example of a flow cytometry print-out is shown in Fig. 1. It shows the result of incubating plasma from a normal control (left) and from a HITT patient (right) with normal type-O blood, in the presence and absence of 0.1 U/ml of heparin. The upper panels show the three platelet populations, separated on the basis of forward and side scatter light: free platelets (bitmap 3), small platelet aggregates (bitmap 2) and large platelet aggregates (bitmap 1). The lower panels show the CD62P expression of the free platelet population from bitmap 3 in the upper panels; see also legend to figure. As shown in this figure, normal plasma caused no apparent increase of CD62P expression in platelets, regardless of the presence or absence of heparin. However HITT plasma induced a clear increase of CD62P expression in the presence of heparin, and only a small increase in its absence.

In the original data the fluorescent intensities of CD62P of the HITT group had a large range. To avoid the problem of unequal variances between groups, we first tested whether the relation between the ± heparin results were both linear and through the origin. This relationship was affirmative, and justified dividing (+) heparin by the (-) heparin values to form a ratio R for each taste. By using the ratio of CD62P fluorescence with/without heparin as explained in Methods, we compared the results from three groups: 12 patients with HITT, 30 patients with HIT, and 65 normal controls. Fig. 2 shows a 2.6-fold increase of mean ratio of CD62P expression (+ heparin/- heparin) in the HITT group, but only 1.25-fold increase for the HIT group. In both groups, these values differed significantly from controls, p < 0.001, as analyzed by t-test. More importantly, the HITT group mean differed significantly from the HIT group, p < 0.001.

A test is considered positive for HIT or HITT if ratio R (defined in Methods) is >2r above the mean R for normal controls. The mean R for n = 65 normal controls was 1.05 ± 0.22. Therefore, if R > 1.50, the test is considered positive. Figure 3 shows a percent-positive analysis. By flow cytometry, 11 of the 12 HITT patients (90%) were positive, but only 5 of the 30 HIT patients (17%) were positive. This difference between HIT and HITT was highly significant, p < 0.0005 by chi-square. These results indicate that this assay may be useful in the clinical discrimination of HITT from HIT. Notice also that when the ELISA method was applied to the plasmas of the same patients for the presence of anti-heparin-PF4 antibodies (see Methods), it failed to distinguish between HIT and HITT: in both groups, 70%–75% of patients were positive in the ELISA (0.70 < p < 0.80, chi-square test).

Discussion

Heparin induced thrombocytopenia with thrombosis (HITT) is the most serious complication of heparin therapy. Laboratory tests able to diagnose HITT would be useful for early detection and prevention of catastrophic thrombosis. Although various assays have been proposed to detect heparin associated antibodies in HIT and HITT (1-3), none so far can discriminate between HITT and HIT. At present therefore, distinction of the two syndromes depends solely on clinical evidence of thrombosis. Boshkov et al. (30) attempted to distinguish between HIT and HITT by measuring activation of coagulation cascade as evidenced by reductions of levels of protein C, antithrombin III, and heparin cofactor II, but no significant difference in these measures between the two groups was seen, nor was there significant difference in degree of thrombocytopenia. Since these two syndromes have profoundly different prognoses, laboratory assay for discriminating them is urgently needed for two main reasons: (i) early detection of HIT, at the beginning of thrombosis or even before thrombosis becomes evident, would allow prevention of progression of thrombotic complication, (ii) in patients needing heparin anticoagulation for life threatening thrombosis, heparin therapy may not have to be discontinued if HITT is unlikely to develop.

The method of the present study shows sufficiently clear differences to provide a basis for differentiating the two syndromes. This study did not address whether this test can diagnose HITT before thrombosis becomes clinically evident. However one patient in the HITT group who developed subclinical silent leg vein thrombosis was positive for HITT by this test. In this patient silent DVT was diagnosed by ultrasound, required as part of a protocol to evaluate a new antithrombotic agent for treatment of HIT/HITT. This observation suggests that this test can detect early asymptomatic stages of HITT and can warn of evolving thrombosis.
thrombosis in asymptomatic patients. Further prospective large scale study will be needed to prove its potential value in this respect.

The basis of our assay is similar to others which detect platelet act-

ivation in response to addition of anti-heparin antibody but differs in several respects which may account for its ability to differentiate HIT from HITT. (i) We employed normal whole blood instead of washed platelets, to avoid platelet pre-activation in vitro. It is known that washing and centrifugation can activate platelets. The use of whole blood instead of isolated platelets approximates more closely in vivo conditions, such as potentially important interactions of platelets with white cells (27). (ii) We used patient plasma instead of serum as the source of antibody, in contrast to most previous work. Sera contains thrombin and other potential activators such as complement which could increase baseline levels of platelet activation. We speculate that the partial activation of washed platelets in the presence of serum could render them more sensitive to the effects of antibody, thereby abolishing the difference we see in response to HIT vs. HITT plasma. This is supported by the evidence of Fig. 3, which shows that only 17% of HIT patients tested positive by the present flow cytometric method. This positive rate is far lower than those reported for washed platelets and sera.

Compared to the ELISA assay for HIT/HITT, the flow cytometric assay is less sensitive in detecting HIT (see Fig. 3) but appears to be highly sensitive and specific for detecting HITT. Therefore, use of both assays is recommended for clinical purposes. Arepaly et al. (29) showed that the sensitivity of ELISA test is about 90%. This was based on finding that 90% of HIT sera that caused C-14-serotonin release were positive in the ELISA method. However the sensitivity of C-14-seroton-
in assay is itself about 90% of clinically defined HIT patients (13). Taking into account these factors, the true sensitivity of the ELISA test is around 80% (0.9 × 0.9 = 0.81) of clinically defined cases of HIT. On the other hand, other workers using similar methods have reported quite different sensitivities. Greinacher et al reported a sensitivity of 33% of 209 patients with a putative diagnosis of HIT, using a PF4/heparin ELISA method (10). This wide variation of sensitivity of ELISA assays reflects in part the limitations of clinical diagnosis of HIT. In the clinical setting of this study, the sensitivity of ELISA was about 70-75% of clinically defined HIT (Fig. 3). Several additional possibilities could result in lower apparent sensitivity in this study. First, we tested only IgG type antibodies and therefore may have missed a small fraction of HIT with IgA or IgM type antibodies; second, the technique of heparin adsorption employed in this study could possibly lower the sensitivity of the test in a fraction of the patient samples; third, we used a fixed amount of PF4 which may not be optimum for all cases.

A major question raised by this study is why plasma from patients with HIT/HITT induces greater expression of CD62P than plasma from patients with HIT. Both groups show similarly positive results by ELISA but a significant difference in induction of CD62P expression when assayed by flowcytometry. Several hypotheses may be suggested. First, the anti-heparin antibody in HIT/HITT may be qualitatively different (e.g., different epitope or subclass). It has been reported that anti-hepa-rin IgA/IgM occur with anti-heparin IgG in some HIT/HITT patients, rarely alone, and that IgA/IgM types are found mainly in HIT, not in HITT (7, and see refs 10, 11 there). These reports do not support the hypothesis that the difference may be due to antibodies of different immunoglobulin classes. Second, the difference may be due to higher titer of antibodies in HIT/HITT. This question might be addressed by dilution experiments in the ELISA and flow cytometric assays. Third, in HIT/HITT additional unknown thrombogenic factors may be generated due to endothelial injury or others resulting in more potent stimuli for release of P selectin from alpha granules. These hypotheses were not investigated in the present study but deserve future investigation.

Fourth, additional antibodies against other antigens may be involved in HITT but not in HIT, inducing greater CD62P expression and promoting thrombosis. In support of this fourth hypothesis, we found antibodies to platelet CD36 (glycoprotein IV) in 4 of 4 HITT patient plasmas (31). Furthermore, incidence of 70% of CD36 antibody was found in the plasma of patients with thrombotic thrombocytopenic purpura (TPP), whose plasma also potently induced expression of CD62P (28, 32). A lower frequency (~20%) of this antibody was observed in plasma of patients with HIT (31). CD36 is a surface receptor of thrombospondin and plays an important role in platelet activation and thrombosis (33). Others have reported heparin-independent autoantibodies to interleukin 8 and neutrophil-activating peptide-2 in both HIT and HITT patients (34). The presence of anti-platelet CD36 antibody was also reported in one suspected case of HITT (35). These data suggest that in HIT/HITT, there may be platelet activating antibodies to antigens other than heparin which could act synergistically. Antibody against CD36 and possibly others (such as other anti-endothelial cell antibodies) may play a role in thrombogenesis of HITT. Other factors predisposing to HITT have recently been suggested. These include aberrant Fc receptors (36, 37), factor V Leiden (38), and association of HITT with recent cardiovascular surgery or cardiovascular complications (30).

The flow cytometric assay introduced in this report appears useful in discriminating HITT from HIT. Combined use of ELISA and flow cytometric assay could assist physicians in predicting outcomes of thrombocytopenias in patients receiving heparin and thus providing a rational basis for prevention and proper therapy for the life-threatening complications of HITT. It remains to be seen if the assay can be useful in detecting HIT before thrombosis becomes clinically evident. Although one case of subclinical thrombosis was detected in this study, a larger-scale prospective study is needed to confirm the clinical utility of this method.

Acknowledgements

This work was supported by, the Jane and Charles Bosco Memorial Fund, the Wallace H. Coulter Research Fund, the Mary Beth Weiss Research Fund, and gifts from Stanley Glaser. We are grateful to Beckman Coulter Corp. for their continuing support. For advice on statistics, we thank Dr. Robert C. Duncan, Professor of Biomedical Statistics, University of Miami.

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Received June 9, 1998 Accepted after resubmission May 17, 1999