The platelet serotonin-release assay

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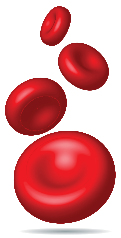

Few laboratory tests are as clinically useful as the platelet serotonin-release assay (SRA): a positive SRA in the appropriate clinical context is virtually diagnostic of heparin-induced thrombocytopenia (HIT), a life- and limb-threatening prothrombotic disorder caused by anti-platelet factor 4 (PF4)/heparin antibodies that activate platelets, thereby triggering serotonin-release. The SRA's performance characteristics include high sensitivity and specificity, although caveats include indeterminate reaction profiles (observed in ~4% of test sera) and potential for false-positive reactions. As only a subset of anti-PF4/heparin antibodies detectable by enzyme-immunoassay (EIA) are additionally platelet-activating, the SRA has far greater diagnostic specificity than the EIA. However, requiring a positive EIA, either as an initial screening test or as an SRA adjunct, will reduce risk of a false-positive SRA (since a negative EIA in a patient with a "positive" SRA should prompt critical evaluation of the SRA reaction profile). The SRA also provides useful information on whether a HIT serum produces strong platelet activation even in the absence of heparin: such heparin-"independent" platelet activation is a marker of unusually severe HIT, including delayed-onset HIT and severe HIT complicated by consumptive coagulopathy with risk for microvascular thrombosis.
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Introduction

Immune heparin-induced thrombocytopenia (HIT) is a prothrombotic adverse drug reaction caused by antibodies that recognize complexes of the cationic chemokine, platelet factor 4 (PF4), when it binds to heparin or certain other polyanions [1,2]. HIT is strongly associated with thrombocytopenia (typically, a ~75% platelet count fall to a median platelet count nadir of ~50 × 10^9/l) and with large-vessel thrombosis (relative risk of thrombosis, 11.6 [95% CI, 6.4–20.8; $P < 0.0001$]) [3], mostly venous (venous:arterial thrombosis ratio, 3-4:1) [4]. However, in severe HIT, patients can develop overt disseminated intravascular coagulation (DIC), with potential progression from macro- to microvascular thrombosis, as exemplified by deep-vein thrombosis evolving to microvascular thrombosis with acral limb loss, especially during concomitant warfarin administration (venous limb gangrene) [5,6].

A key property of "HIT antibodies" is their ability to activate platelets both in vitro [7] and in vivo [8,9]. This occurs when anti-PF4/heparin antibodies of IgG class bind to platelet Fcγ receptors (IgG receptors) [10]. Since the PF4/heparin-IgG complexes are multimolecular [11], two or more Fcγ receptors cross-link, which is a strong platelet activation stimulus [12] that results in platelet shape change and aggregation, α-granule and dense-granule release, and formation of procoagulant, platelet-derived microparticles [8]. HIT is a positive-feedback reaction, since PF4 released from α-granules likely contributes to further activation of platelets [13].

Serotonin is a constituent of platelet dense-granules [14], and measurement of the percentage release of serotonin can be used to quantitate the magnitude of platelet activation induced by a variety of platelet agonists [15,16], including HIT antibodies within patient serum or plasma [17]. However, as discussed subsequently, the key to the platelet serotonin-release assay (SRA) is not the measurement of serotonin-release per se, but rather the handling and preparation of the suspended platelets, methods which differ among laboratories. Thus, one should not assume that the performance characteristics (sensitivity-specificity tradeoffs) reported by one laboratory necessarily apply to others.

History of SRA and Other Platelet Activation Assays for HIT Diagnosis

The history of the development of the SRA is described elsewhere [3,18]. A washed platelet technique utilizing microtiter plates was already in use at McMaster (laboratory of the late Fraser Mustard) for study of platelet activation induced by different platelet agonists. Adapted for testing HIT sera, the assay conditions were fortuitously ideal for detecting HIT antibody-induced platelet activation.

Other functional (platelet activation) assays besides the washed platelet SRA exist. The oldest reported tests for HIT involved classic platelet aggregometry using citrated platelet-rich plasma (PRP), either using patient PRP ("direct") or patient citrated plasma tested against normal donor PRP ("indirect") [19–21]. Unfortunately, PRP-based assays have suboptimal sensitivity (~70–80%), even when performed using optimal donors [22]. Another problem is false-positive results, especially in critically-ill patients [23–25]. For these reasons, PRP-based tests for HIT are rarely performed nowadays. It should be noted that the term "SRA" has also been used when measuring serotonin-release from citrated donor PRP [26]; however, this assay should not be regarded as being similar to the (washed platelet) SRA discussed in this review.

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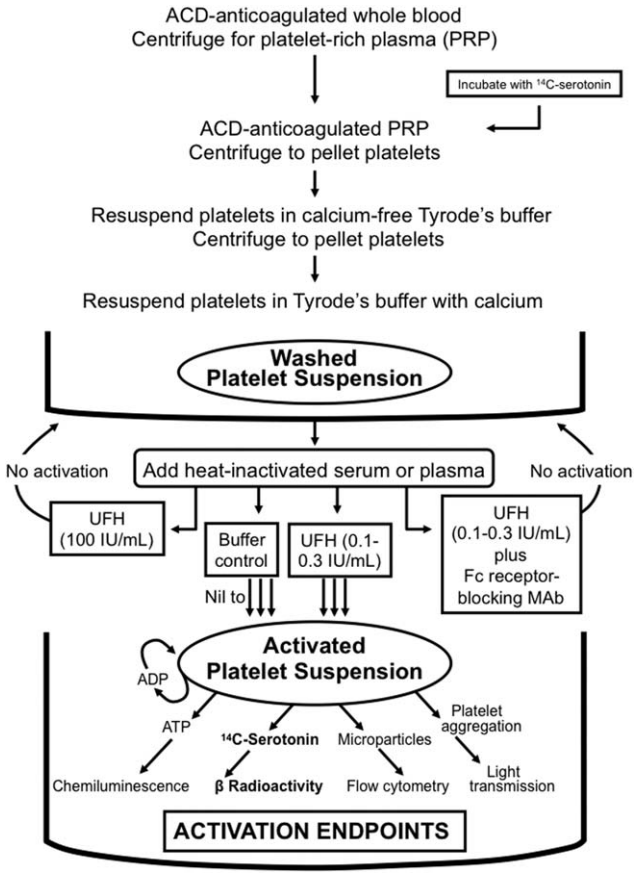

Figure 1. Schematic of washed platelet activation assays, with focus on the SRA. HIT serum (or plasma) causes platelet activation at therapeutic (0.1–0.3 IU/ml) heparin concentrations, but not in the presence of Fc receptor-blocking monoclonal antibody or high (100 IU/ml) heparin concentrations. Platelet activation by HIT serum is potentiated by ADP release from platelet dense granules [36]. Various platelet activation endpoints can be used, including β radioactivity to detect ^{14}C-serotonin. False-positive results can be avoided if typical reaction profiles of non-HIT platelet activation triggers are recognized: e.g., (i) residual thrombin (preferential activation at buffer control versus higher heparin concentrations, i.e., progressively decreasing serotonin-release as the heparin concentration increases from 0 to 0.1 to 0.3 to 100 IU/ml); or (ii) immune complexes (activation at all heparin concentrations–including at 100 IU/ml heparin–with inhibition by Fc receptor-blocking monoclonal antibody). ACD, acid–citrate–dextrose; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ^{14}C, ^{14}Carbon; IU/ml, international units per ml; MAb, monoclonal antibody; PRP, platelet-rich plasma; UFH, unfractionated heparin. Reprinted with modifications, with permission from Warkentin TE Greinacher A, Heparin-Induced Thrombocytopenia, 5th ed, 2013, 272–314, © CRC Press [35].

The heparin-induced platelet activation (HIPA) test, which also uses washed donor platelets, most closely resembles the SRA [27,28]. The major difference is the platelet activation endpoint: for the HIPA, the technologist determines visually the lag time to aggregation in each of the reaction wells. A recent report [29] found that platelet aggregation in the HIPA might occur with some samples that test negative in the SRA, which the authors concluded as indicating potential for false-positive results by HIPA.

Several platelet activation tests that use citrate-anticoagulated whole blood have been described in recent years, including the whole blood multiplate electrode assay [30–32] and a platelet microparticle generation assay [33]. Both types of assays require special equipment, the former a Multiplate® platelet analyzer, and the latter a flow cytometer. To date, definitive large-scale interassay comparisons with the SRA (using identical platelet donors) have not been performed. However, we found that use of platelet-derived microparticles as a platelet activation endpoint gave essentially identical results as our SRA, when washed donor platelets were handled identically in both assays [34].

Technical Aspects

Figure 1 summarizes the SRA [35,36]. Using pedigree blood donors (discussed subsequently), PRP is prepared by centrifugation of acid-citrate dextrose (ACD)-anticoagulated whole blood, to which ^{14}C-serotonin is incubated. After repeat centrifugation, the platelet pellet is resuspended into apyrase-containing, calcium-free Tyrode's buffer. Following repeat centrifugation, the platelet pellet is resuspended into calcium-containing Tyrode's buffer; these platelets are used for the reaction.

Testing is performed using 96-well plates. Patient and control serum (or citrated plasma) that has been heat-treated (30 min at 56 °C to denature thrombin) is used, with the final reaction volume totaling 100 μl, comprised as: test serum (20 μl), heparin/buffer-containing solution (5 μl), and washed platelets (75 μl). The microtiter plate is incubated 60 min at room temperature, shaken (not stirred), with PBS-EDTA buffer added to stop the reaction. The plate is centrifuged to pellet the platelets, and the supernatant is analyzed for radioactivity using a β-counter.

Normal individuals vary greatly with respect to how well their platelets are activated by HIT antibodies (Table I) [37]. Thus, our laboratory only uses selected ("pedigree") platelet donors known to react well in the SRA. The ABO status of the donor is inconsequential.

Reaction Conditions and Positive/Negative Controls

We routinely perform the SRA under the following reaction conditions (all concentrations indicated are final), each in duplicate: (a) buffer control (i.e., UFH 0 IU/ml); (b) UFH 0.1 IU/ml; (c) UFH 0.3 IU/ml; (d) UFH 0.3 IU/ml plus Fc receptor-inhibiting monoclonal antibody, IV.3; and (e) UFH 100 IU/ml. We include a negative control serum, as well as several positive controls, including heat-aggregated IgG and well-characterized strong HIT sera, some of which have been diluted to provide "weak positive" controls. Given the potential for reduced platelet reactivity due to technical or other factors (e.g., recent aspirin use by the donor), it is important to include a weak-positive HIT control serum to ensure that the platelets being used are optimally reactive [37,38]. This also allows us to monitor interassay variability. Although we optimize test sensitivity by routinely using two different pharmacological concentrations of UFH (0.1 and 0.3 IU/ml), it is unusual for a HIT serum to react with only one of the two heparin concentrations (although this is occasionally seen with weak-positive sera).

Characteristic Reaction Profile of Positive HIT Serum

The cut-off between a negative and positive SRA is considered to be 20% serotonin-release [17]. HIT serum usually causes strong platelet activation (>80% serotonin-release) at 0.1 and 0.3 IU/ml UFH, with a smaller proportion causing maximal release between 50 and 79.9%; even fewer SRA-positive sera will cause only weak serotonin-release (20–49.9% release) [39,40]. Thus, the SRA is a "dichotomizing" assay, as samples generally divide into negative or moderately/strongly-positive, with relatively few borderline reactions [39].

A characteristic feature of HIT serum is inhibition of platelet activation (by at least 50% from maximal values) with the addition of

TABLE I. Hierarchically-Ordered Reactions of 10 HIT Sera Tested Against 10 Normal Platelet Donors (i.e., 100 Serum/Platelet Pairs)

	Platelet donors: strongest (P_1) to weakest (P_{10})									
HIT sera (S_1-S_{10})	P_1 84%	P_2 71%	P_3 68%	P_4 54%	P_5 53%	P_6 41%	P_7 40%	P_8 39%	P_9 37%	P_{10} 30%
S_1 85%	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +	+ +	+ + +
S_2 84%	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ + + +	+ + +	+ + +
S_3 69%	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ +	+ + +	+ +	+ +	+ +
S_4 61%	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+
S_5 56%	+ + + +	+ + +	+ + +	+ +	+ + +	+ +	+ +	+	+	+
S_6 51%	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+	+
S_7 44%	+ + + +	+ + +	+ + +	+	+ +	+	+	+	+ +	–
S_8 30%	+ + + +	+ +	+ + +	+	+	+	–	–	–	–
S_9 24%	+ + +	+ +	+	+ +	+	–	–	–	–	–
S_{10} 11%	+ +	+	+	–	–	–	–	–	–	–

Ten randomly selected HIT sera and 10 randomly selected platelet donors are ranked from strongest to weakest (S_1–S_{10} and P_1–P_{10}, respectively), according to the mean percent [^{14}C]serotonin-release values shown when considering all 100 serum-platelet donor pairs. For each individual reaction, the percent serotonin-release is summarized as: 80–100% release, + + + +; 60–79% release, + + +; 40–59% release, + +; 20–39% release, +; <20% release, – . Overall, the reaction profiles for both sera and platelets are *hierarchical*: all negative reactions (<20% release) are in the lower-right part of the table, and the strongest (>80% release) are in the upper-left part of the table. Only platelet donors P_1, P_2, and P_3 would be accepted for use in HIT testing, as they were the only three that yielded positive results in the SRA with all 10 HIT sera, including the weak-positive HIT sera (S_9 and S_{10}), which yielded 20-39% serotonin-release (shown as + in the table). The results are broadly consistent with our laboratory's experience that approximately one-third of randomly tested platelet donors are acceptable for use in HIT testing. Reprinted, with modifications, with permission from Warkentin TE, Hayward CPM, Smith CA, et al., J Lab Clin Med, 1992, 120, 371–379, © Elsevier [37].

high concentrations of heparin (100 IU/ml) [17]. This phenomenon can be explained by the 1:1 to 2:1 molar stoichiometry of PF4:heparin (or PF4:polyanion) required to form the HIT-related antigens [35]: by performing the SRA at 100 IU/ml heparin, the HIT antigens are either disrupted, or do not form, and a negative reaction pattern is seen. Another important feature is that HIT antibody-induced platelet activation can be inhibited by Fc receptor-blocking monoclonal antibody, an observation which helped to show that HIT antibodies activate platelets through cross-linking platelet FcγIIa receptors [10,13]. As discussed later in this review (see section, Positive Buffer Control Reactivity in the SRA), serotonin-release activity without added heparin (i.e., at buffer control) may indicate platelet activation by non-HIT mechanisms or severe subtypes of HIT.

Characteristics that Contribute to SRA Accuracy

Several factors help to explain the excellent operating characteristics of the SRA, including: (a) microtiter assay performed in 96-well plate (opportunity for multiple reaction conditions and controls); (b) divalent cations (calcium, magnesium) in final reaction mixture (retains physiological conditions); (c) reduced concentrations (versus PRP-based assays) of IgG [22,41] and fibronectin [42] (reducing platelet-inhibiting effects of these reactants); and (d) initial wash step performed in presence of apyrase (an enzyme that degrades ADP and ATP, thereby preserving subsequent platelet activation by ADP, an important potentiator of HIT antibody-induced platelet activation) [36]. In addition, the nonspecific platelet-activating effects of heparin

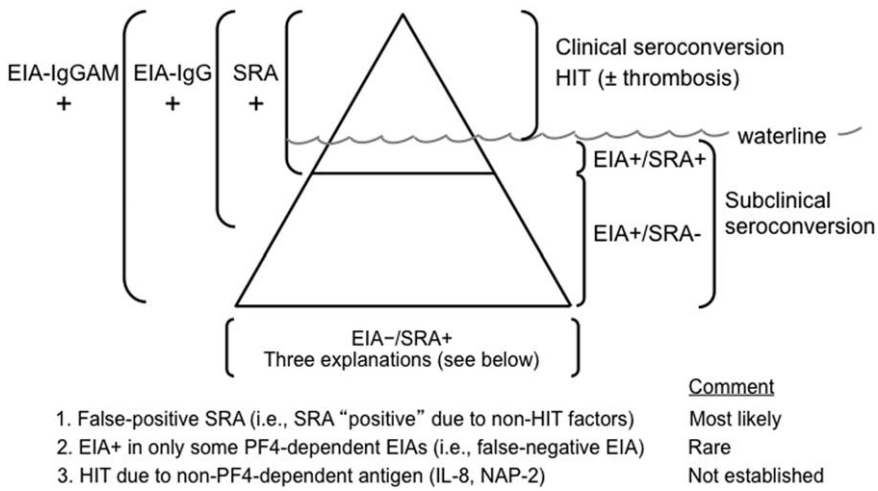

Figure 2. Iceberg model: implications for interpreting EIA-negative/SRA-"positive" reaction pattern. The iceberg model indicates that HIT patients represent the "tip of the iceberg" with (usually) strong positive testing in three assays: SRA, IgG-specific EIA (EIA-IgG), and the polyspecific EIA (EIA-IgGAM). At the bottom of the iceberg, the issue of a negative EIA-IgG and a positive SRA is discussed, listing three possible explanations. The most likely explanation is a false-positive SRA due to HIT-mimicking, non-HIT-related platelet-activating factor(s); this scenario can be avoided by critically assessing any "positive" SRA result in a sample giving an EIA-negative result. A rare (but established [40,49]) explanation is a true-positive SRA with a false-positive EIA, as indicated by an SRA-positive sample that tests negative in a PF4-dependent EIA but that tests positive in at least one other PF4-dependent EIA. The third possibility, which has not been established, is that a HIT-mimicking illness can be caused by non-PF4/heparin-dependent antigens, i.e., interleukin-8 (IL-8) and neutrophil-activating peptide-2 (NAP-2).

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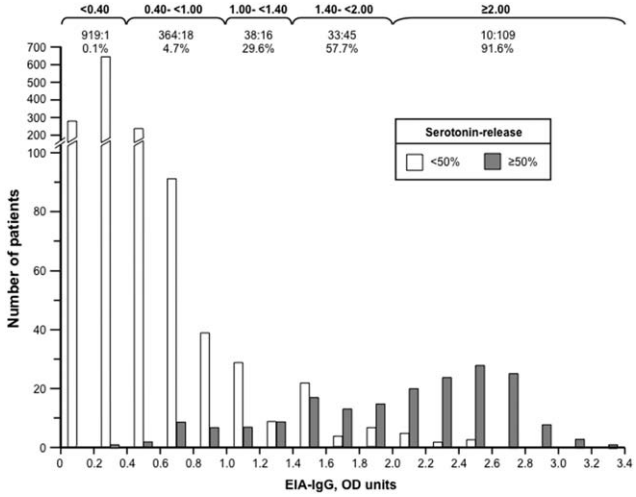

Figure 3. Predictivity of the McMaster Platelet Immunology in-house EIA-IgG for a positive SRA result (>50% serotonin-release, mean at 0.1 and 0.3 IU/ml UFH). For each of five groups of quantitative EIA-IgG data [<0.40, 0.40–<1.00, 1.00–<1.40, 1.40–<2.00, and ≥2.00 optical density (OD) units], the percent of samples yielding a strong-positive SRA result (≥50% serotonin-release) is shown. The probability of a strong-positive SRA result varied considerably, depending upon the magnitude of the EIA result, expressed in OD units. For weak-positive EIA-IgG results (0.40–<1.00 OD units), the probability of a strong-positive SRA result was ~5%. In contrast, for strong-positive EIA-IgG results (OD ≥2.00 units), the probability of a strong-positive SRA result was ~90% (and for EIA-IgG >2.50 units, the probability was 100% (51/51 samples tested). For the EIA-IgG, the probability of a strong-positive SRA result did not reach ~50% until the OD value was approximately 1.20–1.40 units or greater. Reprinted with permission from Warkentin TE Sheppard JI Moore JC , et al., J Thromb Haemost, 2008, 6, 1304-1312, © Blackwell Publishers [39].

[43]—which are believed to be responsible for the phenomenon of nonimmune heparin-associated thrombocytopenia [44]—are not observed using washed platelets (as shown by the inability of normal serum to cause platelet activation in the SRA).

Indeterminate Reactions

We reported in 2008 [45] that ~10% of patient sera tested in our SRA during 2006 and 2007 yielded an initial "indeterminate" reaction pattern, i.e., these samples could not be classified as either positive or negative. When the SRA was repeated, using a newly heat-inactivated patient serum aliquot, approximately 60% then gave a clear positive or negative result; thus, only ~4% of patient sera produced a consistent indeterminate reaction pattern. We further observed that some of these samples are actually very strong positive HIT sera that upon further sample dilution give a clear strong positive reaction profile [45]. In contrast, other sera, particularly those that yield an "immune complex" pattern (i.e., serum-induced platelet activation at all heparin concentrations, including at 100 IU/ml heparin, with inhibition by IV.3), cannot be classified as either positive or negative by SRA [45]. In recent years, the frequency of samples giving repeated indeterminate results that cannot be resolved has fallen to <2%.

Iceberg Model of HIT

We proposed in 1994 the "iceberg model" of HIT [46], which states that patients with HIT represent a small subgroup ("tip of the iceberg") of those who form heparin-dependent antibodies following exposure to heparin. Subsequently, anti-PF4/heparin enzyme-immunoassays (EIAs) were developed to detect HIT antibodies, including both IgG-specific (EIA-IgG) and polyspecific EIAs, the latter detecting any of the three major immunoglobulin classes, IgG, IgA, IgM (EIA-IgGAM). By testing HIT sera with both types of EIAs and with platelet activation assays [47–49], the iceberg model has been refined, including the current concept that HIT is exclusively caused by anti-PF4/heparin antibodies of IgG class (Fig. 2).

A high proportion (at least 99%) of patient sera with a clearly positive SRA (≥50% serotonin-release) have anti-PF4/heparin IgG antibodies detectable by EIA [39,48,49]. An important issue is whether the few SRA-positive/EIA-negative patients represent false-positive SRA results (i.e., serum-induced platelet activation mimicking a HIT reaction pattern that, however, is explained by a non-HIT factor) or an unusual EIA-negative subtype of "true HIT." Indeed, some authors have proposed that antibodies against non-PF4 chemokines (interleukin-8, neutrophil-activating peptide-2) could cause a HIT-mimicking syndrome [50,51]. However, this literature does not provide sufficient clinical information, nor any details of platelet activation results, to establish that a non-PF4-dependent form of HIT exists (further, some of these patients had underlying autoimmune disorders [52]). Unless proven otherwise, we believe that HIT is caused by heparin-dependent platelet-activating antibodies (i.e., patients are SRA-positive) and that these antibodies react against PF4/heparin complexes (i.e., patients are anti-PF4/heparin EIA-positive).

We have seen occasional patients who are SRA-positive but (for unknown reasons) test positive in one particular PF4-dependent EIA but negative in another [40]. Accordingly, we recommend that any patient who has the unusual picture of SRA-positive/EIA-negative status undergo repeat SRA and EIA testing, including testing in another type of EIA.

The EIA as a "Quality Control" Step for the SRA

A corollary to the iceberg model—as inferred by Fig. 2—is that the EIA can be viewed as a "quality control" step that is crucial for avoiding a false-positive SRA report. In other words, we suggest that laboratories be cautious in reporting an SRA as positive if the corresponding PF4-dependent EIA is negative (or only weakly positive), as there is a high chance that the SRA result is false-positive due to a non-HIT-related platelet-activating factor, such as immune complexes (either circulating or formed ex vivo during sample heat-treatment). Another approach used by some laboratories is to follow an algorithm whereby the SRA is only performed if a "screening" PF4-dependent EIA has yielded a positive result.

To illustrate the concept of the EIA representing a quality control step, consider a patient recently investigated for HIT in our laboratory whose serum yielded the following SRA result (%serotonin-release):

a. 81% at 0 IU/ml UFH (buffer control);
b. 72% and 59% at 0.1 and 0.3 IU/ml UFH, respectively;
c. 0% release at 100 IU/ml UFH; and
d. 0% release at 0.3 IU/ml UFH plus Fc receptor-blocking monoclonal antibody.

This "down-sloping" pattern of serotonin-release fulfills the "classic" picture of a "positive" SRA, based upon the two-point serotonin-release profile of >20% release at 0.1 IU/ml UFH but <20% at 100 IU/ml UFH [17]. However, the pattern of progressively declining serotonin-release, as the heparin concentration increases from 0 to 0.1 to 0.3 IU/ml, is *not* characteristic of HIT, and indeed this patient's EIA-IgG tested only weakly positive (0.52 optical density [OD] units [normal, <0.45 OD units]), with only ~13% inhibition (to 0.45) OD units in the presence of high heparin. Thus, if this SRA is classified

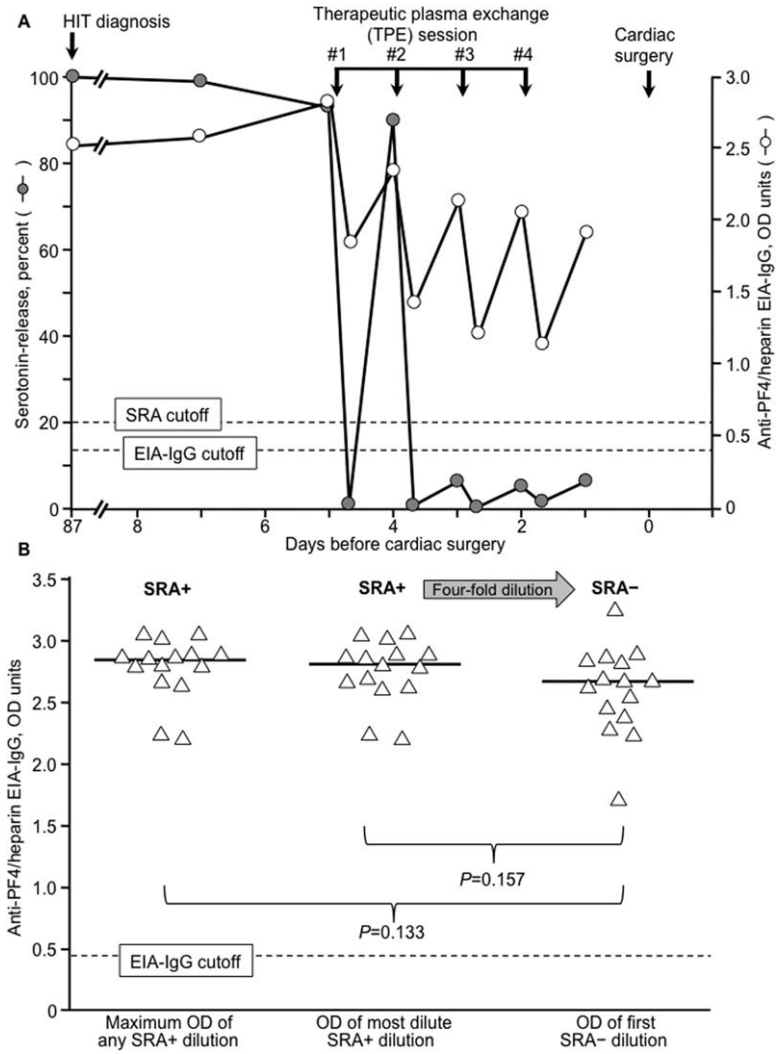

Figure 4. Comparison of McMaster SRA and in-house EIA-IgG with serially diluted samples, either by (A) therapeutic plasma exchange (TPE) or (B) serial sample dilution. (A). Serial SRA and EIA-IgG results in relation to 4 TPE sessions performed on 4 consecutive days (last TPE performed 2 days before cardiac surgery utilizing heparin, performed in a patient who had recent HIT). (B) Comparative studies in the SRA and EIA-IgG using serially-diluted HIT sera. For each of 15 known HIT sera that underwent serial fourfold dilutions—from 1/5 to 1/5,120, and tested in the SRA and EIA-IgG at six different dilutions (i.e., 1/5, 1/20, 1/80, 1/320, 1/1,280, and 1/5,120), we show for each of the 15 HIT sera the: maximum OD level (leftmost data), OD of the most dilute SRA+ sample (middle data), and the OD of the first SRA− diluted sample. The data show an abrupt drop-off in SRA reactivity, with persisting strong reactivity in the respective EIA-IgGs, and with nonsignificant differences in OD values ($P > 0.05$) despite fourfold differences in HIT serum dilution and corresponding markedly different SRA reactivities. (Note that the 1/5 dilution in the SRA—20 μL patient serum or plasma to 80 μL washed platelets with heparin added—represents the standard conditions in the McMaster SRA.) EIA-IgG, enzyme-immunoassay (IgG-specific); OD, optical density; PF4, platelet factor 4; SRA, serotonin-release assay; SRA−, SRA-negative; SRA+, SRA-positive. Reprinted, with modifications, with permission from Warkentin TE, Sheppard JI, Chu FV, et al., Blood, 2015, 125, 195–198. © American Society of Hematology [57].

as "positive" for HIT, this would represent a false-positive SRA. Since some reference labs only perform the classic "two-point" SRA (at 0.1 and 100 IU/ml UFH), such atypical reaction patterns might not be recognized. We believe that including the EIA-IgG (or EIA-IgGAM) as a "quality control" step will help avoid false-positive SRA results. In our experience, these SRA-"positive"/EIA-negative patients usually have a clinical picture that argues against a diagnosis of HIT, e.g., septicemia.

EIA-SRA Interrelationship: Magnitude of EIA Positivity

The strength of a positive EIA—as indicated by OD units—predicts strongly for a positive SRA [39,53] or HIPA [48]. Figure 3 shows that for samples that test weakly positive in our in-house IgG-specific EIA (<1.0 unit of OD), the probability of a positive SRA is ~5%, whereas for samples that yield a strong positive EIA (≥2.00 units), the probability of a positive SRA is ~90%. The explanation for a strong-positive polyspecific EIA with a negative SRA includes high levels of anti-PF4/heparin antibodies of IgA class (a non-platelet-activating class of antibodies). In a patient with a strong-positive EIA and a negative SRA, it is important to exclude a false-negative SRA, which we accomplish by ensuring that the weak-positive control HIT serum reacted satisfactorily in the assay.

The strong association between a higher OD value and a positive SRA helps to explain why several groups of investigators have shown an association between higher OD values and thrombosis risk [54,55]. In essence, the higher OD value indicates a greater probability of "true" HIT, which itself is strongly associated with thrombosis [56].

Importance of HIT Antibody Threshold for Achieving SRA Positivity

Recently, we used therapeutic plasma exchange (TPE) to decrease the titer of HIT antibodies when preparing a patient with recent HIT and persisting SRA-positive status for urgent cardiac surgery [57].

TABLE II. Frequency of Thrombocytopenia (>50% Platelet Count Fall) Among EIA-Positive Patients (Polyspecific or IgG-Specific Assay) who Received Heparin (UFH or LMWH): A Comparison of SRA-Positive Versus SRA-Negative Status

SRA status	Positive in polyspecific EIA (EIA-IgGAM)	Positive in IgG-specific EIA
A. Postorthopedic surgery patients[a]		
EIA+/SRA+	12/24	12/24
EIA+/SRA−	0/58	0/16
P	<0.0001	0.0009
B. Venous thromboembolism patients[ab]		
EIA+/SRA+	4/4	4/4
EIA+/SRA−	0/15	0/6
P	0.0003	0.0048
C. Postcardiac surgery patients		
EIA+/SRA+	4/11	NA
EIA+/SRA−	0/152	NA
P	<0.0001	NA

The data are consistent with the SRA having a high sensitivity for HIT (>95%); the specificity of the SRA depends on the clinical situation, but in most circumstances is at least 95%. Patients in studies "A" and "B" were tested in both the polyspecific and IgG-specific assays. Data to construct this table were obtained from published literature [63–65].

[a] For the data shown, the cut-off for a positive SRA was 20% serotonin-release. For study A (postorthopedic surgery), if instead a 50% serotonin-release cut-off is used, the comparisons (polyspecific EIA) yield similar results: 11/20 vs. 1/62 ($P < 0.0001$), and unchanged data for study B (venous thromboembolism patients).

[b] For the venous thromboembolism study, EIA results were ≥1.0 units of optical density (OD). Reprinted, with permission, from Warkentin TE, Hematology Am Soc Hematol Educ Program, 2011, 2011, 143–149, © The Society [66].

EIA, enzyme-immunoassay; NA, not available; SRA+, positive in the serotonin-release assay; SRA−, negative in the serotonin-release assay.

When we tested the patient's pre- and post-TPE plasma in both the SRA and EIA, in relation to each of the four TPE sessions, we were surprised to see dramatic declines in SRA positivity that occurred with minimal decreases in EIA reactivity (Fig. 4A). To see whether this might be a general phenomenon, we performed serial fourfold dilutions of 15 SRA-positive HIT sera, and found that for each serum, we could identify a diluted sample in which the SRA became negative but the EIA remained strongly positive (Fig. 4B). These studies point to a critical dependence of HIT serum-induced platelet activation to a crucial threshold level of platelet-activating antibodies, so that when an SRA initially becomes negative, either naturally over time [58–60] or through artificial means (serial dilutions or through repeated TPE [57]), HIT antibodies remain readily detectable by EIA. A key clinical observation is that planned heparin re-exposure (e.g., for cardiac or vascular surgery) can be performed when the SRA or HIPA) is negative, even if the EIA remains positive [58–60]. These observations underscore the importance of using a platelet activation assay, rather than the EIA, to judge suitability for heparin re-exposure.

Importance of Using Acute Serum/Plasma for the SRA

As will be discussed in the next section, the SRA is a highly sensitive test for HIT. Indeed, we have shown that both the EIA and SRA are typically strongly positive even at the very beginning of the HIT-related platelet count fall [61]. However, HIT antibodies are remarkably transient [60], and thus it is important that acute serum or plasma (preferably, obtained during thrombocytopenia) is used for testing. We have reported patients with HIT whose degree of EIA and SRA positivity dramatically decreased in association with platelet count recovery, with the SRA reverting to negative in conjunction with platelet count recovery [62]. Other patients may remain SRA-positive for as long as 6 weeks (median) to 3 months [60].

Performance Characteristics of the SRA

In our hands, the SRA has high sensitivity and specificity for the diagnosis of HIT. Indeed, we view laboratory detection of platelet-activating, heparin-dependent antibodies as *sine qua non* for a diagnosis of HIT. We have evaluated the SRA and our McMaster IgG-specific EIA in clinical trials of heparin therapy in which the availability of daily platelet counts allowed for an independent evaluation for presence of HIT. The first opportunity was when we tested in a blinded fashion plasmas from 387 surgical patients who participated in a clinical trial of UFH versus LMWH (enoxaparin) for thromboprophylaxis posthip arthropasty [63]. Using a 50% serotonin-release cutoff, we identified 20 patients who tested SRA-positive. Six (30%) of these 20 patients developed a platelet count fall to less than 150×10^9/L that began on or after day 5 of heparin therapy (i.e., presumed HIT), which was significantly greater than the frequency of thrombocytopenia (2/367 [0.5%]) in the SRA-negative controls (odds ratio, 78.2 [95% CI, 12.0–818.9]; $P < 0.001$). In addition, the two SRA-negative patients had compelling non-HIT explanations for thrombocytopenia (bone marrow replacement by tumor; septicemia secondary to colon perforation).

The frequency of thrombocytopenia was even greater among the SRA-positive patients (at the 50% serotonin-release threshold) if a more sensitive definition of thrombocytopenia (50% drop in platelet count from the postoperative peak platelet count) was used: 11/20 (55%) vs. 4/342 (1.2%), which also indicates a strong association between a positive SRA and presumed HIT (odds ratio, ~100). One of the patients who developed a >50% platelet count fall had a strong-positive EIA but only a weakly positive SRA (20% serotonin-release); this patient also had a urinary tract infection, which perhaps explained the thrombocytopenia. However, if the analysis is performed for an SRA threshold of 20%, and this patient is therefore reclassified as presumed HIT, then the comparison was 12/24 versus 3/342, which also corresponded to a very high odds ratio (~100). The key message is that a positive SRA predicts for an approximate 100-fold greater risk of HIT (by odds ratio) over an appropriate control population, which explains why a thrombocytopenic patient who is investigated for HIT, and who tests SRA-positive, virtually always has HIT.

The value of the SRA is shown by our study [40] of critically ill patients investigated for HIT in a randomized trial ("PROTECT") of UFH versus LMWH (dalteparin). We identified 17 patients who were SRA-positive in this study. When we reviewed these cases, we found a high frequency of thrombosis (10/17 [59%]), and that 16 of the 17 patients had a clinical picture consistent with HIT (4Ts [pretest probability system] score of 4 points or greater); only one patient had a clinical picture that appeared more in keeping with sepsis than with HIT, with the possibility of non-HIT-related subclinical SRA seroconversion.

EIA-Positive/SRA-Positive Versus EIA-Positive/SRA-Negative Status

Table II summarizes data from three studies—two from our group [63,64] and one from Pouplard and Gruel (Tours, France) [65]—in which the frequency of thrombocytopenia (≥50% platelet count fall) are shown for two groups of patients, EIA-positive/SRA-positive and EIA-positive/SRA-negative patients [66]. The studies demonstrate that risk of thrombocytopenia is strongly associated with SRA-positive status, rather than EIA-positive status alone. These observations attest to

TABLE III. Clinical Correlates of Positive Buffer Control Reactivity in the SRA

Feature	References
Unusual HIT syndromes	
Delayed-onset HIT	[71,72]
Fondaparinux-induced HIT	[73,74]
"Spontaneous" HIT	[75–77]
Greater severity of HIT	
Lower platelet counts	[40]
Slower platelet count recovery	[72,78]
Higher frequency of HIT-associated DIC	[71]

the biological significance of anti-PF4/heparin antibodies that are shown to be platelet-activating in vitro. The data are also consistent with the SRA having high sensitivity for detecting HIT, which we believe approaches 100%, assuming that the quality control steps react as expected (particularly, the weak-positive HIT control serum), and acute patient serum (or plasma) is used.

SRA Seroconversion Without HIT

Table II also shows that some SRA positive patients do not develop HIT. This is evident from prospective serosurveillance studies where some patients clearly developed a strong-positive SRA, along with a positive EIA-IgG, and yet did not develop any platelet count fall or thrombotic event [63,65,67]. The explanation for this phenomenon is uncertain, but it could relate to decreased ability of the patient's platelets to respond to HIT antibodies (much the same as some normal donors do not react well to HIT serum). However, to our knowledge, no systematic investigations explaining this phenomenon have been performed. These observations underscore an important tenet of diagnosing HIT, from a clinical-pathological perspective, namely that to diagnose HIT with a high level of confidence, the patient should have a clinical picture that resembles HIT (operationally, a 4Ts score of at least 4 points), with a serological picture that supports HIT (operationally, EIA-positive/SRA-positive status), and without a more compelling diagnosis [38,68].

Use of the SRA in Clinical Practice

As discussed earlier, our assessment is that a positive SRA result (at a 50% serotonin-release threshold, and with a corroborating positive EIA to reduce the risk of a false-positive SRA) increases the probability of the patient having HIT (in relation to the pretest probability) by approximately 100-fold (odds ratio), whereas a negative test effectively rules out the diagnosis. However, the SRA result is almost never available the same day that the test is ordered (even when ordered in Hamilton). Thus, the main purpose of the SRA is not to help with immediate patient management, but rather with ultimately making the correct diagnosis.

Positive Buffer Control Reactivity in the SRA

Our laboratory performs the SRA under several reaction conditions, including at buffer control (i.e., patient serum incubated with test platelets in the absence of heparin). We have observed among

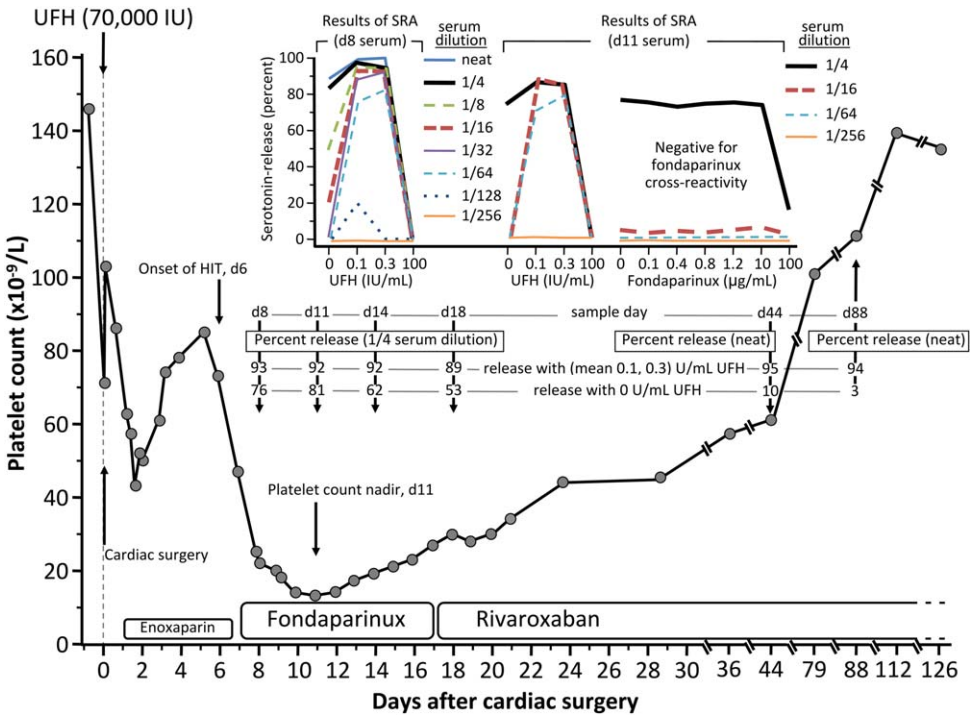

Figure 5. Serial platelet counts following cardiac surgery. Onset of HIT occurred on postoperative day 6. Patient serum obtained on day 8 (d8) and diluted as much as 1/64 tested strongly positive (>80% serotonin-release) for HIT antibodies in the SRA. Patient serum obtained on d11—when thrombocytopenia was maximal—did not show evidence of fondaparinux cross-reactivity: despite strong heparin-dependent serotonin-release using patient serum diluted 1/16 and 1/64, no fondaparinux-dependent platelet activation was seen at these serum concentrations (i.e., no increase in platelet activation, compared with buffer control, was produced by patient serum at any dilution and at any concentration of fondaparinux; moreover, very high fondaparinux concentrations [100 μg/ml] inhibited serum-induced platelet activation). Also shown is percent serotonin-release induced by patient serum (samples obtained on days 8, 11, 14, 18, 44, and 88) in the presence of heparin (mean percent release at 0.1 and 0.3 IU/ml UFH), as well as in buffer control (0 IU/ml UFH). There was an inverse correlation between percent serotonin-release at 0 IU/ml heparin and the severity of thrombocytopenia. These data help to support the degree of buffer control reactivity in explaining the patient's thrombocytopenia. d, day; HIT, heparin-induced thrombocytopenia; IU, international units; UFH, unfractionated heparin. Reprinted with permission from Kopolovic I, Warkentin TE, CMAJ, 2014, 186, 929–933, © Canadian Medical Association [72]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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SRA-positive sera that reactivity at buffer control varies widely, from absent to very strongly positive. Other laboratories have also noticed the frequent occurrence of strong-positive reactivity of the buffer controls in the SRA and HIPA tests [69,70]. We believe that this phenomenon is important, as it is a marker of severe and unusual HIT (Table III) [71–78].

We first reported the phenomenon of high buffer control reactivity in "delayed-onset HIT" (i.e., where HIT begins or worsens after stopping heparin) [71,72]. More recently, we have reported buffer control reactivity to be a feature of fondaparinux-associated HIT syndrome [73,74] (i.e., HIT that begins approximately 1 week after beginning exposure to fondaparinux rather than heparin), as well as "spontaneous" HIT (an illness that is clinically and serologically identical to HIT except without proximate heparin exposure) [75–77]. It appears that the HIT antibodies in these atypical disorders are highly pathogenic and can even activate platelets without the need for any polyanionic drug. Interestingly, in patients with fondaparinux-associated HIT, the pentasaccharide anticoagulant has been reported to enhance patient serum-induced platelet activation (over buffer control), suggesting that performing the SRA with and without pharmacological concentrations of fondaparinux (e.g., 0, 0.1, 0.4 μg/ml) could be helpful in supporting a diagnosis of fondaparinux-associated HIT (note that a very high level of fondaparinux, e.g., 100 μg/ml, as with very high concentrations of UFH, inhibits serum-induced platelet activation, and so the SRA should also be performed at such a high concentration of fondaparinux to increase diagnostic specificity [73,74]).

We have also found that buffer control reactivity predicts for greater pathogenicity of HIT antibodies, including greater magnitude of thrombocytopenia [78], higher frequency of HIT-associated DIC (and associated microvascular thrombotic complications) [71], and longer time to platelet count recovery [78]. We suspect that in the future, some laboratories will provide information on this phenomenon, just as laboratories began to report OD values for the EIA, as it became increasingly clear that the magnitude of a positive EIA result provided important clinical information.

Figure 5 provides an illustration of the value of buffer control reactivity in explaining the clinical course of a patient with delayed-onset HIT postcardiac surgery. By evaluating serial serum samples, we showed a striking inverse correlation between the percent serotonin-release at 0 IU/ml UFH (buffer control) and the patient's platelet count values. The SRA was also helpful in ruling out fondaparinux-dependent antibodies (as no increase in platelet activation in the presence of fondaparinux was observed). This patient case illustrates the role of the SRA in elucidating an unusual case of HIT.

Summary

In our hands, the SRA is a superb test for identifying presence of the pathogenic platelet-activating antibodies that cause HIT. However, the assay is technically challenging, and careful attention to quality control is required to avoid false-negative and false-positive results. Recent attention to the pathogenic significance of positive buffer control reactivity will focus increasing attention to this clinically-relevant aspect of the SRA. Also, by viewing the anti-PF4/heparin EIA-IgG as a "quality control" maneuver will reduce risk of reporting false-positive SRA results.

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