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Activation of platelets by heparin-induced thrombocytopenia antibodies in the serotonin release assay is not dependent on the presence of heparin

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Summary. The serotonin release assay (SRA) tests for antibodies responsible for heparin-induced thrombocytopenia (HIT). By definition, SRA-positive antibodies cause platelet serotonin release *in vitro*, in the presence of low concentrations of heparin, but not with excess heparin. Many SRA-positive sera activate platelets in the presence of saline without drug, either as a result of residual heparin in the specimen, or because of intrinsic features of the HIT antibodies. The present experiments show that neither exhaustive heparinase treatment, nor chromatographic removal of heparin abrogates the spontaneous platelet activation caused by these HIT antibodies. This is the first study to systematically demonstrate that *in vitro* activity of HIT antibodies can be independent of heparin. In addition, T-gel chromatography demonstrated differences among fractions of enzyme-linked-immunosorbent assay (ELISA)-positive HIT antibodies within individual specimens. Certain ELISA-positive fractions had SRA activity while others did not, and the SRA activity was not proportional to HIT antibody ELISA titer. These data suggest that antibodies formed as a result of heparin treatment are heterogeneous, and that some can contribute to the pathogenesis of HIT even when heparin is no longer present.

Keywords: heparin-induced thrombocytopenia, platelets, serotonin release assay, heparin – platelet factor 4 antibody.

Introduction

Heparin-induced thrombocytopenia (HIT) is a potentially life-threatening complication of heparin anticoagulant therapy, characterized by a moderate to severe drop in platelet count after heparin treatment and associated with a paradoxical, high risk of thrombotic complications [1]. HIT is caused by antibodies elicited by a conformational change in the chemokine, platelet factor 4 (PF4), that exposes neoepitopes resulting from its binding to heparin (H) [2,3]. These antibodies bind to H:PF4 complexes via the antigen-specific Fab region, and to platelets at the FcγIIa receptor, resulting in platelet activation and the release of PF4. In the presence of heparin there is continued formation of the H:PF4 complexes, initiating a cycle of platelet activation believed to be responsible for both the thrombocytopenia and the hypercoagulable state that leads to HIT-associated thrombosis [4].

The pathogenic mechanism as described above, however, does not adequately explain the occurrence of HIT without continuing or subsequent exposure to heparin, such as occurs in patients developing delayed-onset HIT, with thrombosis or thrombocytopenia days or weeks after their heparin exposure [5–7]. This mechanism does not explain the observation that cessation of heparin alone is not sufficient to circumvent the progression of HIT pathology [8]. It has been proposed that some populations of HIT antibodies cross-react with PF4 alone or in complex with endogenous heparin-like compounds present on the platelets or endothelial cells [5,9,10].

In addition to the clinical observations, there are laboratory findings of interest. It is known that certain HIT sera elicit platelet activation in the absence of heparin *in vitro* in the heparin-induced platelet activation (HIPA) assay [11, 12] and in the serotonin release assay (SRA) [13]. In the HIPA studies, the spontaneous response to HIT sera was abrogated by procedures to clear heparin contamination from the specimens [11,12]. While residual serum heparin is a possible explanation for spontaneous SRA activity [14], there are no data to directly support this. In fact, heparin-independent SRA activity has been demonstrated in sera from 'delayed HIT' patients who had not received heparin for more than 5 days [5]. The present studies were conducted to determine whether residual heparin in HIT specimens contributes to spontaneous SRA activity.

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Materials and methods

HIT specimens

All HIT specimens used in these studies, obtained under Institutional Review Board approval, were from patients clinically suspected of having HIT whose diagnosis was confirmed by a positive SRA result, or from patients plasmapheresed for treatment of HIT thrombosis [15]. Of specimens with spontaneous SRA activity, only those with sufficient volume available for experiments could be included in the study.

Reagents and buffers

Unfractionated porcine sodium heparin was obtained from American Pharmaceutical Partners Inc., (Los Angeles, CA, USA). Buffer reagents were from Sigma Chemicals (St Louis, MO, USA). The three SRA buffers included acid-citrate dextrose (ACD; 66.6 mM citric acid and 0.85 mM sodium citrate, 5.5 mM dextrose, pH 4.5), calcium and albumin-free Tyrode's (CAF; 137.5 mM NaCl, 2.7 mM KCl, 12 mM NaHCO$_3$, 0.4 mM NaH$_2$PO$_4$, 2 mM MgCl$_2$, 4.4 mM HEPES, 5.5 mM dextrose, pH 6.2), and albumin-free Tyrode's (AFT; 137.5 mM NaCl, 2.7 mM KCl, 12 mM NaHCO$_3$, 0.4 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 4.4 mM HEPES, 5.5 mM dextrose, pH 7.4).

Heparinase-I from IBEX (Montreal, Canada) was buffered in 50 mM Tris HCl, 175 mM NaCl, pH 8.4. A bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford, IL, USA) was used for protein determinations. Heparin was assayed as anti-factor Xa (FXa) activity using bovine FXa (Stago Diagnostica, Asnieres, France) and Spectrozyme FXa chromogenic substrate (American Diagnostica, Inc., Greenwich, CT, USA) in 50 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH 8.4 as previously described [16].

Serotonin release assay

The SRA [14] was performed as follows. Blood from a normal donor, known to be reactive in the assay, was collected in ACD. Platelet rich plasma was prepared (80 g, 15 min) and incubated with 50 nCi mL^{-1} (^{14}C) serotonin (Amersham, Piscataway, NJ, USA) for 45 min at 37 °C. The platelets were washed with CAF buffer containing 1.5 U mL^{-1} apyrase (Grade III; Sigma), then suspended in AFT buffer. Test specimens were heat-inactivated (56 °C, 45 min) then centrifuged (800 g, 10 min) prior to assay, to neutralize residual thrombin or other enzymes. The radiolabeled, washed platelets were incubated in a V-bottom polypropylene 96-well plate with the test specimen, and vehicle (saline) or unfractionated heparin at final concentrations of 0.1 or 100 U mL^{-1}. After 60 min of incubation at room temperature on a rotating shaker, EDTA (4% in saline) was added to stop the reaction. The assay plates were centrifuged and radioactivity in the supernatant was counted (LKB 1209 Rack Beta Liquid Scintillation Counter; Perkin Elmer, Downers Grove, IL, USA) in test wells and also

in wells of untreated, labeled platelets (background) and platelets lysed with 10% Triton-X (total release).

An SRA-positive specimen causes 20% or greater serotonin release in response to 0.1 U mL^{-1} heparin, with inhibition of that response in the presence of 100 U mL^{-1} heparin. Negative specimens show < 20% serotonin release at all heparin concentrations. Specimens that cause > 20% serotonin release at all heparin concentrations are classified as indeterminate.

ELISA for HIT antibody

The GTI-PF4 ELISA assay (Genetics Testing Institute, Waukesha, WI, USA) was used according to manufacturer's instructions.

Heparinase-I digestion

HIT specimens were incubated 45 min at 37 °C, or for 24, 42, or 65 h at 30 °C with a final concentration of 1.0 U mL^{-1} heparinase-I [17,18]. The longer, more exhaustive heparinase digestions were conducted at 30 °C, to assure stability of the enzyme; 90% of heparinase-I activity remains after 72 h at 30 °C [18]. The activity of this enzyme to cleave unfractionated heparin in pooled human plasma was verified using the chromogenic anti-FXa assay [16].

After heparinase-I digestion, samples were heat inactivated (56 °C, 45 min) prior to testing using SRA. For controls, specimens in buffer only were incubated in parallel with heparinase-I treated specimens.

Thiophilic adsorption chromatography (T-gel)

Immunoglobulin-G (IgG) fractions of a normal human serum (NHS) and of SRA-positive sera were isolated using T-gel purification (Pierce, Rockford, IL, USA). Centrifuged (10 000 g, 20 min) 1 mL specimens containing 87 mg mL^{-1} K$_2$SO$_4$, were applied to a 5-mL column of T-gel adsorbent and eluted with binding buffer (0.5 M K$_2$SO$_4$, 50 mM Na$_2$HPO$_4$, pH 8.0), followed by 12 column volumes of elution buffer (50 mM Na$_2$HPO$_4$, pH 8.0). The protein content and ELISA titer of each 3-mL column fraction were determined to locate the enriched IgG peak. Sodium azide was eliminated from buffers to avoid interference with platelet function in subsequent SRA testing.

Aliquots containing 1.5 μg of titrated-heparin (^{3}H-heparin) (American Radiochemical Corp., St Louis, MO, USA) (8.7 μCi per 0.1 mg mL^{-1}) were added to 1 mL of normal and of SRA-positive serum prepared for T-gel fractionation. Aliquots (100 μL) of each fraction were counted for radioactivity (as described for SRA) to determine the pattern of elution of heparin from T-gel.

Statistical analysis

Results using non-treated and heparinase-I-treated HIT specimens were analyzed by paired *t*-test using MS Excel software; $P < 0.05$ was considered significant.

Results

HIT specimens and platelet activation in the SRA in the absence of added heparin

In the year 2000, 934 specimens were tested by SRA at Loyola University Medical Center. Of 41 that tested positive, 31 (75.6%) caused activation of platelets without the addition of heparin to the SRA incubation (Fig. 1). Results for subsequent years were similar at 66.6% (38 of 57) in 2001, 67.2% (39 of 58) in 2002, 66.1% (37 of 56) in 2003, and 69.8% (37 of 53) in the year 2004.

Inactivation of unfractionated heparin by heparinase-I

To remove any possible heparin contamination in the patient specimens, heparinase-I was used. As small heparin chains retain anticoagulant activity, and can cross-react in HIT antibody assays [19], it was important to use heparinase-I to degrade any residual heparin to its dissaccharide components. The anti-FXa assay of heparin, sensitive to the activity of low molecular weight heparin fragments [17], demonstrated the complete inactivation of heparin concentrations ≤ 0.25 by 1 U mL^{-1} of heparinase-I (Fig. 2).

Effect of heparinase-I treatment of HIT specimens on SRA activity

Heparinase-I treatment to clear specimens of possible residual heparin did not prevent platelet activation by HIT sera in the presence of saline alone. For both enzyme-treated and non-treated specimens, each SRA showed a positive serotonin release that was increased by, but not dependent upon, addition of a low concentration of heparin, along with the negative response to a high concentration of heparin. Figure 3 shows the SRA response of each HIT specimen assayed before
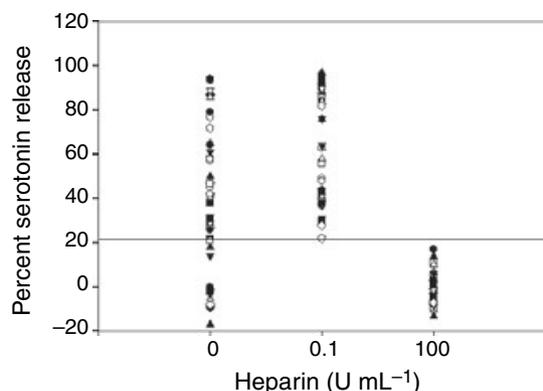

Fig. 2. Heparinase-I cleaves unfractionated heparin to fragments that no longer have biologic activity. Incubation of unfractionated heparin with heparinase-I (45 min, 37 °C), results in loss of activity measured by the anti-factor Xa activity assay, which would detect residual low molecular weight heparin fragments. Less than 0.5 U mL^{-1} heparin would be expected in clinical samples.

and after heparinase-I treatment. With some specimens, there was slightly less SRA activity after enzyme incubation, but the results were still > 20% serotonin release, the cutoff for a positive response. Thus, there was no example in which heparinase-I treatment significantly decreased or eliminated the SRA positive response.

PF4, released from platelets when blood clots, is abundant (5–10 µg mL^{-1}) in patients' sera [20]. Thus any heparin present in a specimen would likely be in the form of H:PF4 complexes, or HIT antibody-H:PF4 immune complexes, which could explain the positive response in the SRA saline control incubation. As heparin in complex with serum components in HIT specimens could be less susceptible to enzymatic cleavage than heparin alone, extended incubation times were employed in the heparinase-I experiments. Results were compared between time points to determine whether there was any incremental increase in heparinase-I effect after 45 min, 24, 45, or 65 h. There was no evidence of a time-dependent trend to indicate that extended heparinase-I treatment diminished the platelet activation response (Fig. 3). Even after 65 h of heparinase-I incubation, HIT specimens continued to cause platelet activation without addition of heparin to the SRA.

Elution of ^{3}H-heparin from T-gel

In order to study HIT antibodies in isolation from serum components, including any residual heparin, HIT specimens were fractionated by T-gel chromatography. T-gel binds IgG of any sub-type, allowing other serum components to flow through the column. Subsequent elution yields a peak enriched in IgG. Protein measurements of each fraction defined the flow-through fractions and the bound/eluted IgG peak fractions.

Fig. 1. Many serotonin release assay (SRA)-positive sera cause platelet activation without addition of heparin to the assay. The graph depicts percent of serotonin release from donor platelets incubated with SRA-positive sera in the presence of saline, 0.1 or 100 U mL^{-1} heparin. Each set of symbols depicts the SRA results of one specimen. Of 41 SRA-positive specimens, 31 (75.6%) caused spontaneous platelet activation, i.e. > 20% serotonin release with saline alone.
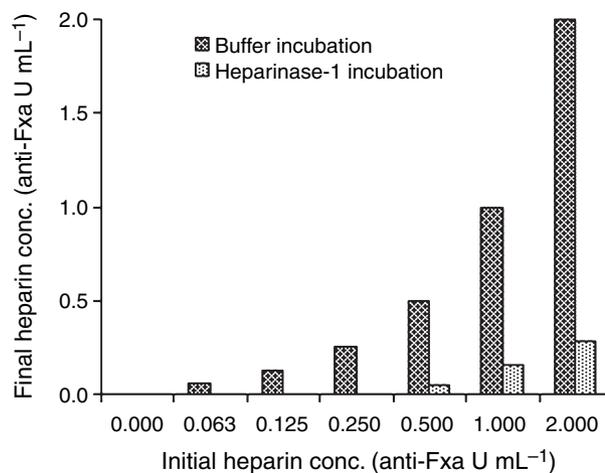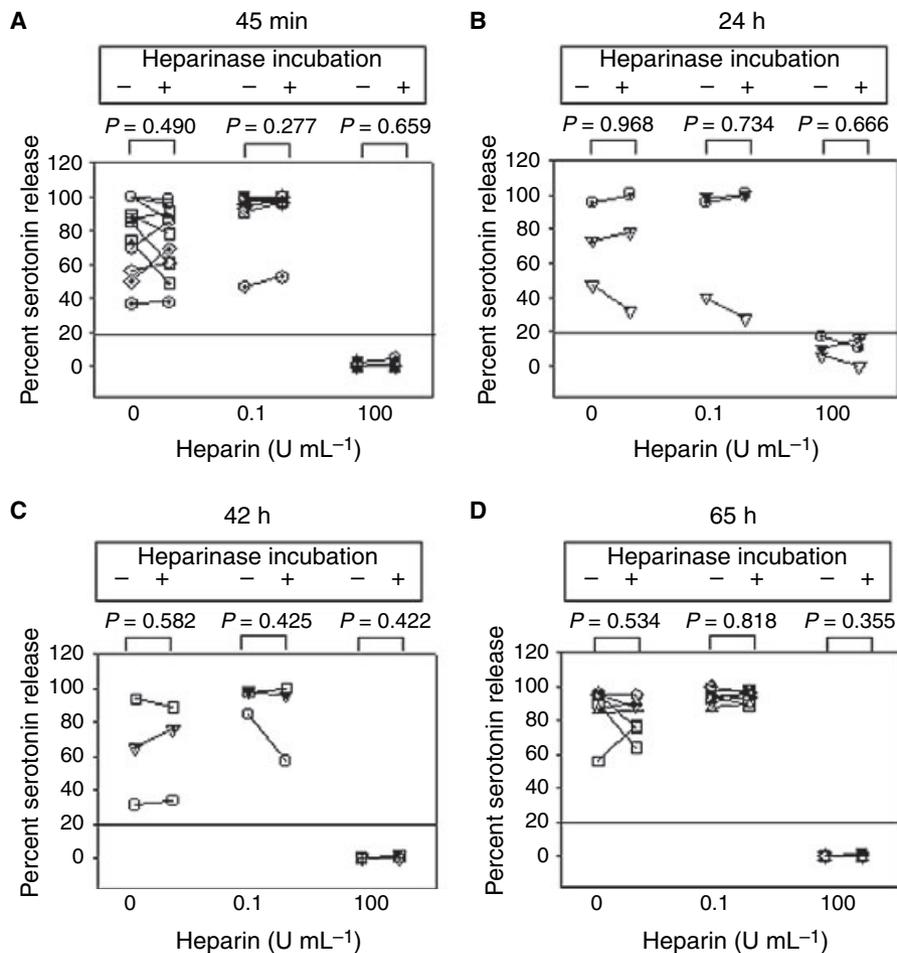
Fig. 3. Pre-incubation with heparinase-I (1 U mL^{-1}) did not abolish activation of platelets by serotonin release assay-positive sera in the absence of heparin. Each symbol represents one heparin-induced thrombocytopenia specimen; lines indicate the change in serotonin release because of incubation of specimens with heparinase prior to assay. Platelet response to all specimens remained positive ($> 20\%$ serotonin release) after heparinase treatment for 45 min ($n = 11$), 24 h ($n = 3$), 42 h ($n = 3$) or 65 h ($n = 7$). Paired *t*-tests showed no significant difference between responses with or without heparinase treatment.

One normal and one HIT serum were mixed with 75 000 cpm of ^{3}H-heparin prior to the T-gel chromatography. Figure 4 shows the protein elution profile, compared with the location of the eluted radioactivity. With both specimens, radiolabeled heparin eluted in the flow-through peak. Radio-activity in a 100-µL aliquot of each bound/eluted (IgG) fraction was < 15 cpm over background. These results demonstrate that T-gel chromatography effectively separated residual heparin from serum immunoglobulins in the presence as well as in the absence of HIT antibodies.

Effect of isolation of HIT IgG fractions by T-gel chromatography on SRA activity

T-gel elution profiles of four HIT specimens that caused platelet activation in the presence of saline alone are shown in Fig. 5. In addition to the protein concentration, these graphs also show the HIT antibody ELISA titer for each fraction. ELISA positive fractions (OD > 0.4) were localized to the IgG

peaks. Because of the difference in sensitivity between protein determination (0.1 mg mL^{-1}) and the HIT ELISA, many fractions had significant antibody titer even though no protein was detectable. Table 1 shows the SRA response of fractions eluted from the T-gel column. For each specimen, at least two of the IgG fractions were positive by SRA. Isolation of these SRA-positive IgG fractions from heparin and from other components of the specimen, did not abolish the platelet activation response that occurred in the absence of exogenous heparin.

In addition, T-gel fractionation demonstrated heterogeneity among HIT antibody fractions within individual specimens. While all of the T-gel fractions of specimens B and C (Table 1) had high HIT ELISA titer, only two fractions from each specimen tested positive in the SRA. All fractions from specimen A, on the contrary, were SRA positive even though two were negative (OD < 0.4) in the ELISA. Comparison of ELISA titers with percent serotonin release for individual fractions shows that SRA activity is not always proportional to
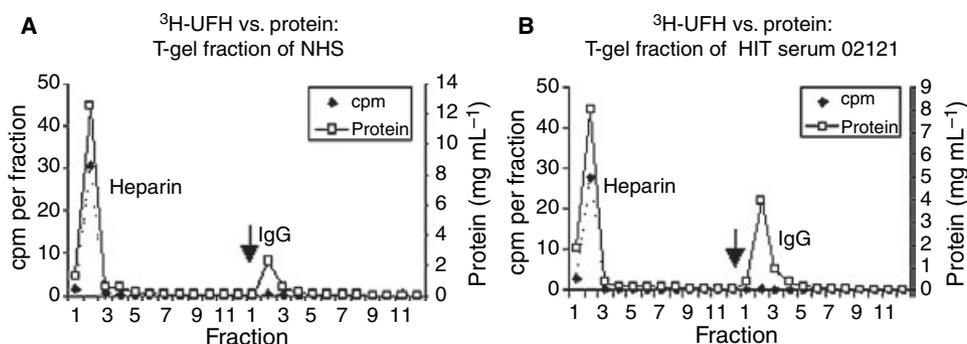
Fig. 4. Elution pattern of tritiated heparin vs. protein from T-gel. Profiles show the radioactivity (◆) and protein concentration (□) of flow through fractions and fractions eluted from T-gel after buffer change (↓). Heparin was not retained by the T-gel column or bound to heparin-induced thrombocytopenia (HIT) immunoglobulin-G (IgG). The radiolabel eluted separately from the IgG peak of normal (A) or HIT (B) serum.
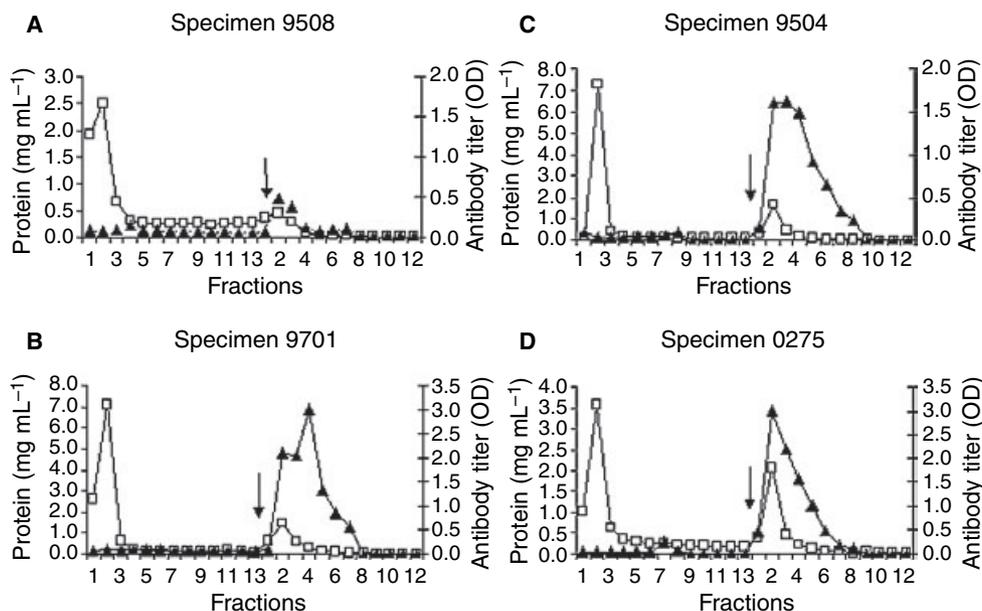

Fig. 5. T-gel fractions from heparin-induced thrombocytopenia (HIT) specimens. Profiles show the protein concentration (□) of flow through fractions and fractions eluted from T-gel after buffer change (↓). HIT antibody fractions were identified by ELISA titer (▲), and were localized to the bound/eluted immunoglobulin-G (IgG) fractions. As a result of the sensitivity of the HIT ELISA, detectable levels of antibody eluted over several fractions, even when total IgG protein was below the limits of detection (0.1 mg mL^{-1}) of the bicinchoninic acid assay.

antibody titer as would be expected with a monoclonal antibody population.

Individual IgG fractions from the same specimen also differed in the ratio of heparin-independent to heparin-dependent serotonin release. There were fractions in three of the four specimens that caused less platelet activation in the presence of 0.1 U mL^{-1} heparin than with saline alone.

Discussion

The present experiments were undertaken to demonstrate that the activation of platelet serotonin release by HIT antibody is not always dependent on the presence of exogenous heparin. While no heparin anti-FXa activity could be detected in the clinical specimens, it was imperative to further prove that the

studies were performed in a heparin-free environment. To accomplish this HIT specimens were incubated with heparinase-I, or fractionated using T-gel chromatography to isolate HIT IgG. Both strategies were used with replicate HIT specimens, and tested with multiple SRA platelet donors. The present results validate preliminary evidence from a study which had used a single, pooled HIT antibody specimen [21].

Potszch [12] demonstrated that heparinase treatment could prevent false positive aggregation tests because of heparin contamination. In that study, heparinase-I at room temperature for 15 min was sufficient to eliminate spontaneous platelet aggregation in five of eight sera of HIT patients tested during or shortly after cessation of their heparin treatment. In our study with 11 specimens, heparinase-I incubation was carried out at 37 °C for 45 min, with even more exhaustive incubations up to

Table 1 Serotonin release assay (SRA) results for immunoglobulin-G (IgG) fractions (Fr.) from T-gel chromatography of heparin-induced thrombocytopenia (HIT) specimens described in Fig. 5

		Percent serotonin release		
Specimen	HIT antibody titer (OD)	Saline	0.1 U mL^{-1} (UFH)	100 U mL^{-1} (UFH)
A. 9508				
Fr. 2	0.520	93	72	0
Fr. 3	0.361	76	83	6
Fr. 4	0.115	69	37	2
B. 9701				
Fr. 2	2.094	0	5	0
Fr. 3	2.064	87	77	8
Fr. 4	3.000	82	79	9
C. 9504				
Fr. 2	1.599	3	13	0
Fr. 3	1.607	41	63	7
Fr. 4	1.483	80	59	10
D. 0275				
Fr. 2	3.000	60	96	0
Fr. 3	2.126	63	93	0
Fr. 4	1.566	89	97	0

UFH, Unfractionated heparin.
Each specimen yielded at least two fractions that fulfilled the criteria for positive SRA response. Activation of platelets by the IgG fraction was not dependent on addition of exogenous heparin. Platelet activation was not proportional to antibody titer. Some ELISA positive fractions did not cause platelet activation, and some SRA-positive fractions had negative ELISA titer (OD < 0.4).

65 h. In contrast to what has been observed in aggregation assays [11,12], the serotonin release response with heparin-depleted specimens remains equal to the pre-treatment response.

A second strategy to test for SRA activity of HIT antibodies in the absence of any residual heparin, was to isolate IgG from the specimens by T-gel chromatography. Isolated IgG fractions from each HIT specimen continued to cause spontaneous platelet activation in the SRA. Even using the two protocols in tandem, that is, heparinase-treating isolated T-gel fractions, or using T-gel to isolate IgG from heparinase-treated specimens, did not abolish the SRA activity of the HIT IgGs before addition of heparin to the assay (data not shown).

In the aggregation study cited above [12] short heparinase-I incubation, testing of isolated IgG, or re-testing 3–5 days after heparin withdrawal, abrogated the HIT serum-induced platelet aggregation that had occurred without addition of heparin. As residual heparin gives rise to spontaneous platelet activation measured by the aggregation assay, it was logical to assume that it also explains spontaneous platelet activation in the SRA. However, there is no support from the present study, or evidence from previous literature, to indicate that heparin contamination can also explain the activation of platelets by SRA-positive specimens tested with saline alone.

The immunogenic H:PF4 complex may generate antibodies with varying extents of cross-reactivity and differences in target specificity and affinity between antibody populations [22–24]. It has already been documented that not all H:PF4 ELISA positive specimens cause a response in *in vitro* platelet

activation tests [25]. In the present studies, T-gel fractionation demonstrated heterogeneity of HIT antibodies within individual specimens [26]. This is the first report showing that a single specimen can be separated into multiple fractions of H:PF4 ELISA-positive antibodies only some of which have SRA activity. Also, in one specimen there were fractions with high SRA activity but low levels of antibody as measured by ELISA. Some T-gel fractions in three of the four specimens studied caused less platelet activation in the presence of 0.1 U mL^{-1} heparin than with saline alone, a result rarely seen in SRA of whole sera. Because T-gel chromatography depletes PF4 from the IgG fractions (< 1 ng mL^{-1}: M. Prechel, unpublished data), there is a shift in the H:PF4 ratio toward heparin excess in SRA incubations using T-gel fractions instead of serum. Because of the low PF4 level, the threshold between a stimulatory vs. inhibitory level of heparin could occur at a relatively low heparin dose, and could vary depending on the specificity and titer of H:PF4 antibodies in the fractions.

Many studies have established that specific complexes of unfractionated heparin and PF4 are critical for stimulation of HIT antibody formation, and that the antibodies bind with highest affinity to these complexes [3]. Yet, once formed, a significant proportion of HIT antibodies also target PF4 without heparin [22,27]. In general, HIT antibodies do not recognize native, soluble PF4, but rather PF4 that is modified by binding, exposing conformational neoepitopes [28,29]. But, this conformational change is not exclusive to association with unfractionated heparin. PF4 associated with heparan sulfate on endothelial cells [30], bound to other sulfated polysaccharides [31], or immobilized on agarose beads or polystyrene plates, is recognized by many HIT sera [29]. As PF4 also binds on the surface of platelets [32], it is reasonable that certain populations of HIT antibody may specifically recognize PF4 bound on platelet membrane glycosaminoglycans in the SRA, even in the absence of added heparin. The platelet-bound PF4:HIT antibody immune complex could then activate other platelets via the FcγIIa receptor. This mechanism could explain how HIT sera, cleared of heparin contamination, still cause spontaneous platelet activation *in vitro*.

With the trend toward shorter hospital stays, many patients are no longer being monitored for 5–8 days after heparin exposure, the interval when HIT antibodies might first develop. It is imperative to consider that risk factors resulting in PF4 expression on cells within the vascular system could generate the antigenic target for HIT antibodies. Such conditions could sustain the immune response initiated by the H:PF4 complex and play a role in the pathology of HIT even in the absence of heparin. Two groups have described cases of delayed-onset HIT, where previously hospitalized patients with heparin exposure presented with thromboembolic complications days to weeks after their initial discharge [5,6]. Both studies reported that lack of information on the patient's recent heparin exposure, or lack of awareness of HIT and its proper treatment, led to potentially disastrous clinical outcome when patients were re-administered heparin for the current thrombotic problem. Data from Warkentin's study of 'delayed HIT'

patients, with onset of symptoms 5 or more days after their last heparin exposure, show these patients' antibodies to have higher heparin-independent platelet activation in the SRA than those of patients with typical HIT onset [5]. This observation is consistent with the results of our study, indicating that the presence of exogenous heparin is not always required for the pathogenic activity of HIT antibodies.

In this study, experiments were conducted with multiple, individual HIT specimens. Our data show that activation of platelet serotonin release by HIT specimens in the absence of exogenous heparin is a common occurrence. Heparinase-I digestion of the HIT specimens and/or isolation of HIT immunoglobulins from the specimens did not abrogate this heparin-independent response, indicating that the response is not an artifact of heparin contamination. Individual SRA positive specimens were fractionated by T-gel chromatography into heterogeneous fractions that varied in HIT ELISA titer, and in the heparin-dependent and heparin-independent platelet activating activity. These results demonstrate that HIT antibodies are comprised of polyclonal populations of immunoglobulins, some of which have physiologic activity even in the absence of exogenous heparin.

Acknowledgements

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