Heparin-Induced Thrombocytopenia: Laboratory Studies

By John G. Kelton, David Sheridan, Aurelio Santos, James Smith, Karen Steeves, Carol Smith, Claudia Brown, and William G. Murphy

This report describes studies into the pathophysiology of heparin-induced thrombocytopenia. The IgG fraction from each of nine patients with heparin-induced thrombocytopenia caused heparin-dependent platelet release of radiolabeled serotonin. Both the Fc and the Fab portions of the IgG molecule were required for the platelet reactivity. The platelet release reaction could be inhibited by the Fc portion of normal human or goat IgG, and patient F(ab')2, but not F(ab')2 from healthy controls. These results suggested that the Fab portion of IgG binds to heparin forming an immune complex and the immune complexes initiate the platelet release reaction by binding to the platelet Fc receptors. To directly challenge this hypothesis, we preincubated the serotonin-labeled platelets with the monoclonal antibody against the platelet Fc receptor (IV.3). This monoclonal antibody completely inhibited the release reaction caused by heparin and patient sera, as well as heat-aggregated IgG, but did not block collagen or thrombin-induced platelet release. Heparin-dependent platelet release also could be inhibited in vitro by the addition of monocytes and neutrophils, but not by red cells, presumably because the Fc receptors on the phagocytic cells have a higher binding affinity for IgG complexes than do platelets. Platelets from patients with congenital deficiencies of specific glycoproteins Ib and IX (Bernard-Soulier syndrome) and Ib and Ila (Glanzmann's thrombasthenia) displayed normal heparin-dependent release indicating that the release reaction did not require the participation of these glycoproteins. These studies indicate that heparin-induced thrombocytopenia is an IgG-heparin immune complex disorder involving both the Fab and Fc portion of the IgG molecule.

HEPARIN-INDUCED thrombocytopenia is a common complication of heparin therapy occurring in up to 5% of patients who receive this drug.1 For most patients the thrombocytopenia does not cause serious morbidity and rapidly resolves once the heparin has been discontinued.1,3 However, for a small subset of patients, the thrombocytopenia is complicated by acute arterial thrombosis that can lead to myocardial infarction, cerebral vascular accidents, and limb ischaemia.1,4,7 Some of these patients die as a consequence of these side effects of heparin.1 Despite the importance of heparin-induced thrombocytopenia, its pathophysiology has not been well studied. Several years ago, heparin-dependent platelet aggregation was described in these patients.8 Subsequently, Cines et al demonstrated that patient IgG could initiate heparin-dependent serotonin release from normal platelets9 as well as bind to endothelial cells.10 Other investigators have reported heparin-dependent binding of patient IgG to normal platelets.11 However, the mechanism of interaction of the IgG with the patient platelets has not been characterized.

In a previous study, we demonstrated that although heparin-dependent platelet aggregation is a not a sensitive test for heparin-induced thrombocytopenia,12 the clinical usefulness of the test can be improved by measuring heparin-dependent platelet release at two different heparin concentrations.13 In the current study, we used this assay to study sera from nine patients with heparin-induced thrombocytopenia. We found that the platelet release reaction required both the Fab and the Fc portion of the IgG molecule. Platelet release could be blocked by the addition of human or animal Fc, and a monoclonal antibody (IV.3) that binds to the platelet Fc receptor. These studies indicate that heparin-induced thrombocytopenia is caused by the binding of heparin-IgG immune complexes to platelet Fc receptors.

MATERIALS AND METHODS

Patients with heparin-induced thrombocytopenia. Serum samples were collected from nine patients with heparin-induced thrombocytopenia. In every patient, thrombocytopenia developed following initiation of heparin treatment and other causes of thrombocytopenia were excluded. Three serum samples were from patients with heparin-induced thrombocytopenia plus acute arterial thrombosis. One sample was from a patient who redeveloped thrombocytopenia after inadvertently receiving heparin following an initial episode of thrombocytopenia. All of these patient samples were strong heparin-dependent initiators of platelet release. Control sera included samples from healthy laboratory personnel and patients who were receiving heparin but who did not become thrombocytopenic. Heparin-dependent platelet release. Heparin-dependent platelet release was measured using test sera (or the IgG preparations that will be described), two different heparin preparations (bovine and porcine), and 14C-serotonin-labeled platelets.11 Platelet-rich plasma from two healthy, non-thrombocytopenic individuals receiving no medications was isolated and labeled with 14C-serotonin (Amersham, Oakville, Ontario) at a concentration of 0.1 μCi/mL of platelet-rich plasma. The platelets were washed once and resuspended in Tyrode's albumin at a final concentration of 3.0 x 10^7/μL. Twenty microliters of patient or control serum (or IgG fraction) was added to 75 μL of 14C-serotonin-labeled platelets (225 x 10^7/L,

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To this solution, 5 μL of bovine or porcine heparin (Hepalean; Ontario Canada Ltd., Toronto) or buffer was added to produce final heparin concentrations that ranged from 0.05 to 100 U/mL. The platelet suspension was gently mixed for 60 minutes at 22°C, at which time 100 μL of 0.5% EDTA, was added. The platelets were pelleted by centrifugation, and the supernatant radioactivity measured. All tests were performed in triplicate, and the platelet release of serotonin was reported as a percentage of the total platelet radioactivity, after correction for background radioactivity, as described previously. A positive test for heparin-induced thrombocytopenia has been defined as >20% release at 0.1 units heparin, and <20% release at 100 units heparin. Positive controls included serotonin release caused by heat aggregated IgG.

Preparation of IgG, F(ab')2, and Fc. Patient and control IgG was purified by one of two different techniques: ion exchange chromatography using DEAE Affi-gel (Bio-Rad, Mississauga, Ontario) or affinity chromatography using staphylococcal protein A beads (Bio-Rad). Purity was confirmed by analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and was >95%. The IgG prepared by either technique was equally reactive and produced unimodal heparin-dependent release reactions identical to the respective intact serum.

Goat IgG was purified from 50% ammonium sulfate precipitated goat sera followed by affinity chromatography using staphylococcal protein A.

F(ab')2, and Fc fragments were prepared from human and goat IgG using pepsin and papain digestion as described previously. Monomeric IgG was digested with papain (100:1, wt:wt) (Sigma Chemical Co, St Louis) to produce Fe, or pepsin (30:1) to yield F(ab')2 fragments. The purity of the preparations was confirmed by analytical SDS-PAGE (12%) and was >90% for all.

The monoclonal antibody (IV.3) against the platelet Fc receptor was a generous gift from Dr C.L. Anderson (Columbus, Ohio).

The use of platelets from patients with congenital deficiencies of certain platelet glycoproteins. Washed platelets were obtained from a patient with the Bernard-Soulier syndrome and a patient with Glanzmann's thrombasthenia. The patient with the Bernard-Soulier syndrome had larger than average sized platelets, a life-long history of bleeding, a bleeding time of >30 minutes, normal levels of plasma von Willebrand factor, but absent platelet aggregation to ristocetin. Complete absence of platelet glycoproteins Ib and IX was confirmed by radioimmunoprecipitation and immunoblot studies. The patient with Glanzmann's thrombasthenia also had a life-long bleeding disorder characterized by a bleeding time of longer than 30 minutes and typical platelet aggregation responses. This patient had complete absence of platelet glycoproteins Ib and IIa confirmed by radioimmunoprecipitation and immunoblot studies. Neither patient was receiving any medication nor had received recent platelet transfusions. Platelets from these patients were labeled with 14C-serotonin and heparin-dependent release tested as described using sera from two patients with heparin-induced thrombocytopenia plus the previously described control sera. These studies were performed on two different occasions and gave identical results each time.

Determination of the portion of IgG required for heparin-dependent platelet release. These studies were performed using both platelet aggregation and platelet release as endpoints. For the aggregation studies, the platelets were prepared in an identical fashion as the release studies except the relative ratio of the platelets and patient IgG was proportionally increased to allow evaluation of aggregation. In preliminary experiments that used the strongly reactive sera from the patients with heparin-induced thrombocytopenia, identical results were obtained whether aggregation or release was used as an endpoint. Because the release reaction gave a quantitative endpoint and used smaller volumes of reagents, platelet serotonin release assays were performed in the subsequent studies.

To investigate the component of IgG required for the platelet release reaction, 20 μL of purified human or goat Fc giving a final concentration of 0.15, 0.36, and 0.73 mg/mL was mixed with 75 μL of platelets (225 x 10^9/L, final) for 15 minutes at room temperature. To this mixture, was added 20 μL of purified patient IgG (five different patients) or control IgG (88 to 44 μg), and 6 μL of heparin (final concentration, 0.1 or 100 U/mL). Platelet release was determined as described. Controls for these and subsequent experiments included heat aggregated IgG, which acts as an IgG-immune complex, sera from healthy laboratory personnel, and sera from patients who had received heparin but had not become thrombocytopenic. Each experiment was performed in triplicate.

To investigate the requirement of the Fab portion of the molecule for the heparin-dependent release reaction, 20 μL of patient or control F(ab')2 (30 to 66 μg) was incubated with 75 μL of 14C-serotonin–labeled platelets (225 x 10^9/L, final) for 15 minutes at 22°C. To this mixture was added 20 μL of patient IgG (160 and 80 μg) and 6 μL heparin (0.1 U/mL, final), and the percent release determined.

The use of monoclonal anti-platelet Fc receptor antibody. To test whether the platelet Fc receptor was involved in the reaction, we preincubated test platelets with a monoclonal antibody directed against the platelet Fc receptor. This monoclonal (IV.3) has previously been shown to bind to a 40 kd platelet glycoprotein and prevent IgG immune complexes from binding to the Fc receptor. Aliquots of the 14C-serotonin–labeled platelets (300 x 10^9/L) were preincubated at 22°C for 30 minutes with one of the following: (1) monoclonal antibody (IV.3) to give a final dilution of 1/200 of the ascites fluid; (2) monoclonal antibody (IV.3) to give a final dilution of 1/2,000 of the ascites fluid; (3) nonimmunized ascites fluid to give a final dilution of 1/200; or (4) buffer. The different platelet suspensions were subsequently used for the 14C-serotonin release reaction using sera from two different patients with heparin-induced thrombocytopenia. Positive controls included heat-aggregated IgG, thrombin, and collagen.

Inhibition of heparin-dependent platelet release by other cells. Because the binding of immune complexes to cells depends on the affinity of the cell Fc receptor, we investigated the effects of monocytes and neutrophils (which carry high affinity Fc receptors) and red cells (which do not carry Fc receptors) on the heparin-dependent platelet release reaction. Preparations with a >90% purity of neutrophils, monocytes, and red cells were prepared as described. To mimic the physiological ratio of platelets to neutrophils and monocytes, we used 225 x 10^9/ L platelets, 3.4 x 10^9/L neutrophils, and 0.25 x 10^9/L monocytes (all final concentrations). In other experiments, the ratios of platelets to neutrophils and monocytes were varied from the physiological level. These experiments were performed by incubating 25 μL of platelets (225 x 10^9/L, final) and 25 μL of monocytes and neutrophils to produce the following final concentration of cells (x 10^9/L): 0.25 and 3.4, 0.08 and 1.1, and 0.01 and 0.1, respectively. To this mixture test serum and heparin were added. In another experiment the monocytes and neutrophils were replaced by buffer, or 25 μL of red cells (118 x 10^9/L). Heparin-dependent platelet release was then determined as described.

Measurement of in vitro immune complexes. Sera from three of the patients with heparin-induced thrombocytopenia, sera from non-thrombocytopenic heparin patients and sera from healthy controls were either left untreated or mixed with buffer or treated with heparin at a concentration of 0.01, 0.1, 1.0, and 100 U/mL (final). After 30 minutes of incubation at 37°C, an aliquot was tested for the presence of immune complexes using a PEG precipitation assay. An
aliquot was tested for its ability to cause release of ¹⁴C-serotonin from test platelets using the previously described assay.

RESULTS

All nine patients had heparin-induced thrombocytopenia diagnosed using clinical criteria. Each patient had thrombocytopenia (range 20 to 90 x 10⁹/L) that developed following initiation of heparin therapy. In eight of the patients, the thrombocytopenia developed following treatment with heparin of porcine origin and in one patient the thrombocytopenia followed treatment with bovine heparin.

Platelet aggregation and release studies. Serum from each of the nine patients had heparin-dependent platelet serotonin release at pharmacological concentrations (0.1 U/mL, final) but not at high concentrations of heparin (100 U/mL, final). In four patients, heparin-dependent platelet aggregation was also investigated, and all had heparin-dependent aggregation at 0.1 units of heparin per milliliter, but not at 100 units of heparin per milliliter (final).

In every patient, the IgG fraction caused the release reaction to occur using either porcine or bovine heparin. The heparin-dependent release reaction could be diluted to nonreactivity with buffer. There was no correlation between the titer of the activity and the clinical outcome. The platelet release reaction (either percent release, or the ability of the sera to be titred with buffer) was not different for patients with isolated thrombocytopenia compared with patients with heparin-induced thrombocytopenia plus arterial thrombosis. No patient (50 tested) receiving heparin who was not thrombocytopenic gave positive results. No other “control” gave positive results in the subsequently described studies.

The use of platelets from patients with congenital deficiencies of certain platelet glycoproteins. Platelets from the patient with Glanzmann's thrombasthenia and the patient with the Bernard-Soulier syndrome had similar heparin-dependent release as the control platelets (Table 1) indicating that the interaction of the heparin-IgG complexes with the platelets did not require platelet glycoproteins Ib, IX, IIb, and IIIa for the platelet release reaction.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Final Heparin Concentration (%)</th>
<th>Control platelets</th>
<th>Bernard-Soulier platelets</th>
<th>Glanzmann's thrombasthenia platelets</th>
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<td>0.1</td>
<td>100</td>
<td>0.1</td>
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<td>Control</td>
<td></td>
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<td>14</td>
</tr>
<tr>
<td>Patient 2</td>
<td>&lt;10</td>
<td>89</td>
<td>&lt;10</td>
<td>10</td>
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The heparin-dependent platelet ¹⁴C-serotonin release (mean of triplicate tests expressed as a percent of maximal platelet release) for two patients with heparin-induced thrombocytopenia using platelets from a patient with Bernard-Soulier syndrome (absent glycoproteins Ib and IX) and a patient with Glanzmann’s thrombasthenia (absent glycoproteins Ib and IIIa). Control sera (described in the Materials and Methods section) consistently resulted in <20% of ¹⁴C-serotonin release.

Determination of the portion of IgG required for heparin-dependent platelet release. The heparin-dependent release reaction could be inhibited by Fc prepared from the IgG fraction of healthy normals who had not been exposed to heparin. Animal (goat) Fc behaved identically to human Fc. The reaction was of a competitive nature and the greatest inhibition was observed at the highest ratio of Fc (human or goat) to patient IgG (Fig 1). The platelet release reaction could also be inhibited by the F(ab')₂ portion of IgG prepared from a patient with heparin-induced thrombocytopenia. Preincubation of platelets and heparin with patient F(ab')₂ (30 μg) reduced heparin-dependent platelet release caused by intact IgG (from a patient with heparin-induced thrombocytopenia) from 100% to 35%. Increasing the concentration of patient F(ab')₂ to 66 μg further reduced the mean percent release to 6% (triplicate tests). Lowering the concentration of intact patient IgG by 50% resulted in complete inhibition of the release reaction by either concentration of patient F(ab')₂. In contrast, F(ab')₂ prepared from healthy volunteers neither inhibited nor augmented the heparin-dependent release reaction.

The use of monoclonal anti-platelet Fc receptor antibody. Confirmation that the platelet release reaction initiated by heparin and IgG from these patients was mediated by the platelet Fc receptor was provided by the experiments using the monoclonal antibody (IV.3), which binds to the platelet Fc receptor. A 1:2,000 dilution of ascites

![Fig 1](https://example.com/figure1.png)

**Fig 1.** The effect of increasing concentrations of Fc or protein buffer (B) (human serum albumin at the same protein concentration) displayed along the abscissa on the platelet release of ¹⁴C-serotonin (ordinate) induced by the IgG fraction from four patients (●) with heparin-induced thrombocytopenia. Each point is the mean of triplicate studies. The percent release at the 0 concentration of Fc was adjusted to 100% to facilitate comparison among the patients. Fc-G indicates purified Fc prepared from goat IgG. Fc-H indicates purified Fc prepared from human IgG. ΔIgG indicates that heat aggregated IgG was used as a positive control for immune complex mediated platelet release.
fluid containing the monoclonal antibody reduced heparin-dependent platelet release to below background levels (Fig 2). The monoclonal antibody also completely blocked platelet release initiated by heat aggregated IgG immune complexes (Fig 2).

Thrombin-induced platelet release was not prevented by this monoclonal antibody. Similarly, a 1:100 dilution of collagen produced 40% platelet release when added to a buffer and platelet mixture, 30% release when added to the control ascites and platelet mixture, and 23% release when added to the monoclonal IV.3 platelet mixture. A 1:200 dilution of collagen produced 36% release (buffer), 28% release (control ascites), and 22% release (IV.3 ascites).

Inhibition of heparin-dependent platelet release by other cells. The studies investigating the effects of the addition of cells carrying high affinity Fc receptors (monocytes and neutrophils) to 14C-serotonin-labeled platelets before the addition of the patient sera and heparin are shown in Table 2. The addition of increasing concentrations of monocytes and neutrophils progressively reduced heparin-dependent platelet release. When the ratio of platelets to monocytes and neutrophils was adjusted to physiological proportions, heparin-dependent platelet release was totally inhibited. In the experiment in which red cells only were added to the platelet suspension, the platelet-release reaction was not affected.

Measurement of in vitro immune complexes. The three serum samples from three different patients with heparin-induced thrombocytopenia all displayed typical patterns of platelet serotonin release with release observed at 0.1 and 1.0 U/mL of heparin, but no release at 100 U/mL (final). None of these patient samples nor any of the controls had PEG detectable immune complexes at any heparin concentration.

### DISCUSSION

Heparin-induced thrombocytopenia is a common and potentially serious adverse reaction to heparin. As many as 5% of patients treated with heparin will become thrombocytopenic, usually on day 5 to 10 of therapy.1 For most patients the thrombocytopenia itself does not result in serious morbidity. However, for a small subset of patients, acute arterial thrombosis complicates the thrombocytopenia.1-3 These patients can have a heart attack, stroke, or acute arterial obstruction to one of their limbs. Despite the high frequency of this complication and its potentially serious impact, the pathophysiology of heparin-induced thrombocytopenia has not been well studied. The observation that the majority of patients with heparin-induced thrombocytopenia have elevated levels of platelet-associated IgG12,13 and in vitro studies by Cines et al demonstrating the interaction of IgG with platelets suggests that immune mechanisms could contribute to the disorder.9

Many patients with this syndrome have heparin-dependent platelet aggregation, although this reaction is not diagnostic of heparin-induced thrombocytopenia.8,12 But by measuring heparin-dependent platelet release at two heparin concentrations, the test can be made both sensitive and
specific. In this report we describe the application of this test to investigate the pathophysiology of heparin-induced thrombocytopenia. Using the heparin-dependent platelet release reaction as the measurement endpoint, we demonstrated that in every patient tested, platelet release was caused by the IgG in the patient's serum. The in vitro reactivity was identical whether the heparin was of porcine or bovine origin. In addition, we observed no correlation between the clinical outcome and the in vitro reactivity. Essentially, identical reactions were noted in patients who had mild heparin-induced thrombocytopenia and patients who had heparin-induced thrombocytopenia plus acute arterial thrombosis. These results suggest that idiosyncratic patient factors determine whether the patient has thrombocytopenia or thrombocytopenia plus arterial thrombosis.

The aim of the next part of the study was to try and determine which part of the platelet membrane was involved in the platelet release reaction. Using platelets from patients with congenital absences of certain glycoproteins, we noted normal heparin-dependent release (Table 1) indicating that platelet glycoproteins Ib, IIb, IIIa, and IX were not required. This observation is consistent with subsequent studies that identified the platelet Fc receptor as being required for the platelet release reaction. Previously we have shown that the binding of IgG immune complexes to platelets, measured either by a direct binding study or by immune complex induced platelet release does not involve any of these platelet glycoproteins. Our observation that glycoproteins Ib and IX are not involved supports the report of other investigators who noted that platelets from patients with the Bernard-Soulier syndrome will aggregate when mixed with patient sera and platelets from patients with heparin-induced thrombocytopenia.

We have previously reported that patients with heparin-induced thrombocytopenia have a unimodal curve of platelet release: low concentrations of heparin trigger the release reaction, whereas high concentrations of heparin do not. This type of reaction is typical of immune complex binding to an Fc receptor with the complex being disrupted by the addition of increasing concentrations of antigen, in this case heparin. But while the observation is consistent with an immune complex interaction, it does not prove it. Indeed, an identical reaction might occur if the antigen (heparin) was displacing heparin-IgG complexes from the platelet membrane, as has been previously shown to occur for malaria-induced thrombocytopenia. Therefore, we did further experiments to characterize the IgG-heparin-platelet interaction. We found that both portions of the IgG molecule were required for platelet reactivity. F(ab')2 from a patient with heparin-induced thrombocytopenia, but not control F(ab')2, was capable of blocking the release reaction in a concentration-dependent fashion. We postulate that the F(ab')2 prevented the platelet release reaction by disrupting the IgG-heparin immune complexes. The demonstration that purified human or animal (goat) purified Fc was capable of blocking the release reaction in a concentration-dependent fashion also was consistent with our hypothesis that heparin-IgG immune complexes triggered platelet aggregation and release by binding to the platelet Fc receptors (Fig 1). To further challenge this possibility, we performed blocking experiments using a monoclonal antibody (IV.3) that binds to the platelet Fc receptor. Very low concentrations of this monoclonal antibody prevented platelet activation and release initiated by IgG immune complexes made by heat precipitation. In contrast, this monoclonal antibody did not prevent thrombin and collagen-induced platelet release, although for uncertain reasons release was reduced slightly.

Thus, four independent lines of evidence including (1) the antigen (heparin) inhibition experiments, (2) the human and animal Fc inhibition experiments, (3) the monoclonal anti-Fc receptor inhibition experiments, and (4) inhibition by patient F(ab')2, all indicated that the mechanism of the platelet release reaction was mediated by the binding of heparin-IgG immune complexes to the platelet Fc receptors. Because the binding affinity of IgG immune complexes for the platelet Fc receptor is low, one would anticipate that in vivo heparin-IgG complexes would preferentially bind to the higher affinity Fc binding sites on the monocytes and neutrophils and not to the Fc receptors on the platelets. To investigate this possibility, we added increasing numbers of neutrophils, monocytes, or red cells to the serotonin-labeled platelets before adding patient sera and heparin. When monocytes and macrophages (Table 2) were added to the test platelets in a proportion that simulated physiological proportions, heparin-dependent platelet release was totally prevented. This observation suggests that other, as yet unidentified factors, could facilitate the binding of the heparin-IgG immune complexes to the platelet Fc receptors. Other observations also remain unexplained. For example, although the heparin-IgG complexes were shown to initiate the platelet release reaction by binding to the platelet Fc receptors, we were not able to document the presence of immune complexes in the sera samples using the polyclonal anti-Fc receptor inhibition experiments, and (4) inhibition by patient F(ab')2, all indicated that the mechanism of the platelet release reaction was mediated by the binding of heparin-IgG immune complexes to the platelet Fc receptors. Thus, four independent lines of evidence including (1) the antigen (heparin) inhibition experiments, (2) the human and animal Fc inhibition experiments, (3) the monoclonal anti-Fc receptor inhibition experiments, and (4) inhibition by patient F(ab')2, all indicated that the mechanism of the platelet release reaction was mediated by the binding of heparin-IgG immune complexes to the platelet Fc receptors.

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